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THE THERAPEUTIC VALUE OF HYPOCHLOROUS ACID.

BY

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(From the Lister Institute of Preventive Medicine, London.)

In October of last year I communicated to the British Medical Journal[1] some experiments on the neutralizing effects of hypochlorous acid on diphtheria toxin, and drew attention to the resistance the animal body offers to large quantities of the solution used.

During the last nine months various investigators have dealt with the hypochlorites as antiseptics—Lorrain Smith, Murray Drennan, Rettie and Campbell,[2] Dakin,[3] Schütze[4]—and also given the results of treatment by intravenous injection—Lorrain Smith, Ritchie and Rettie,[5] and Brand and Keith,[6] in cases of puerperal infections; Fraser and Bates,[7] in cases of toxaemia consequent to gas gangrene.

In this paper I publish further and more detailed experiments to determine the antitoxic and antiseptic power of hypochlorous acid *in vivo.*

It has been shown by Dakin[3] that *in vitro* staphylococci suspended in saline are killed within two hours by sodium hypochlorite, 1 in 500,000, but that when serum is present a concentration of 1 in 1,500 is needed.

It has been thought, therefore, important to prove experimentally (1) which is the highest concentration of HClO tolerated by animals, and (2) what is the lowest concentration producing an antiseptic or antitoxic effect.

Rabbits of about 2 kilos in weight and therefore with approximately 90 c.cm. blood were used. The HClO

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solution employed was eusol with a hypochlorous acid concentration of 0.5 per cent., physiological saline being used in place of water and $CaCl_2$ added on account of its antitoxic properties; it is prepared as follows:

To 800 c.cm. physiological saline add 10 grams bleaching powder and 10 grams boric acid, shake vigorously, allow to stand for two or three hours, then filter and add to the filtrate 2 grams of chemically pure calcium chloride. The solution now contains HClO in a strength of between 0.5 and 0.6 per cent.; saline is added until the required concentration of 0.5 per cent. is reached. Owing to its instability the solution must not be used longer than three days after preparation.

While 20 c.cm. of this HClO solution, giving a concentration of about 1 in 1,100 in the blood of the animal, were generally well tolerated, 10 c.cm. proved to be entirely harmless, and the concentration of about 1 in 2,000 that resulted appeared, moreover, to be near the limit of efficiency; 5 c.cm. doses (about 1 in 3,800) seldom led to beneficial results.

Attention may be drawn to the fact that a great number of toxins of bacterial origin are considered to be of an albuminoid nature (Brieger and Fraenkel,[8] Brieger and Boer,[9] Etienne Burnet[10]); it is possible, therefore, that hypochlorous acid may neutralize toxins by coagulating and eventually precipitating them.

The following tables show the efficacy of 10 c.cm. doses of the HClO solution in certain artificially produced infections and toxaemias.

TABLE I.—*Diphtheria Toxin.*

Rabbit.	Dose of Toxin Injected Subcutaneously	Quantity of HClO Solution Injected Intravenously.				Results.
		Immediately	After 24 hrs.	After 48 hrs.	After 72 hrs.	
1	10 L.D.	10c.cm.	10c.cm.	—	—	Animals remain healthy.
2	10 L.D.	10c.cm.	10c.cm.	—	—	
3	10 L.D.	10c.cm.	10c.cm.	—	—	
4	10 L.D.	—	10c.cm.	10c.cm.	—	After 5 days paralysis of the legs.
5	10 L.D.	—	10c.cm.	10c.cm.	—	
6	10 L.D.	—	10c.cm.	—	10c.cm.	
Control	10 L.D.	—	—	—	—	Dead after less than 48 hours.

TABLE II.—*Tetanus Toxin.*

Rabbit.	Dose of Toxin Injected Subcutaneously	Quantity of HClO Solution Injected Intravenously.			Results.
		After 24 hrs.	After 48 hrs.	After 72 hrs.	
1	0.1 c.cm.	10 c.cm.	—	10 c.cm.	Tetanus.
2	0.1 c.cm.	10 c.cm.	10 c.cm.	—	Healthy.
3	0.1 c.cm.	10 c.cm.	10 c.cm.	—	
Control	0.1 c.cm.	—	—	—	Tetanus.
		SERIES B.			
4	0.25 c.cm.	10 c.cm.	10 c.cm.	—	After 4 days tetanus; recovered after 10 days.
5	0.25 c.cm.	10 c.cm.	10 c.cm.	—	
6	0.25 c.cm.	10 c.cm.	10 c.cm	—	
Control	0 25 c.cm.	—	—	—	Dead after 4 days
		SERIES C.			
7	0.50 c.cm.	10 c.cm.	10 c.cm.	10 c.cm.	Dead after 7 days.
8	0.50 c.cm.	10 c.cm.	10 c.cm.	10 c.cm.	Dead after 11 days.
9	0.50 c.cm.	10 c.cm.	10 c.cm.	10 c.cm.	Dead after 7 days.
10	0.50 c.cm.	10 c.cm.	10 c.cm.	10 c.cm	Healthy.
11	0.50 c.cm.	10 c.cm.	10 c.cm.	10 c.cm.	Dead after 8 days.
Control	0 50 c.cm.	—	—	—	Dead after 4 days.

TABLE III.—*Staphylococcus pyogenes aureus.*

Rabbit.	Number of Micro-organisms Injected Intravenously.	Quantity of HClO Solution Injected Intravenously.			Results.
		Immediately.	After 24 hrs.	After 48 hrs.	
1	50 million	10 c.cm.	10 c.cm.	—	Animals remain healthy.
2	50 ,,	10 c.cm.	—	10 c cm.	
3	50 ,,	10 c.cm.	—	10 c.cm.	
Control	50 ,,	—	—	—	Dead after 48 hrs.
		SERIES B.			
4	100 million	10 c.cm.	10 c.cm.	—	Animals remain healthy.
5	100 ,,	10 c.cm.	10 c.cm.	—	
6	100 ,,	10 c.cm.	10 c.cm.	—	
7	100 ,,	10 c.cm.	10 c.cm.	—	
8	100 ,,	10 c.cm.	10 c.cm.	—	Dead after 10 days.
Control	100 ,,	—	—	—	Dead after 24 hrs.

Table IV.—*Bacillus perfringens.*

Rabbit.	Number of Micro-organisms Injected Subcutaneously.	Quantity of HClO Solution Injected Intravenously.			Results.
		After 24 hrs.	After 48 hrs.	After 72 hrs.	
1	50 million	10 c.cm.	10 c.cm.	—	Animals remain healthy.
2	50 ,,	10 c.cm.	10 c.cm.	—	
3	50 ,,	10 c.cm.	10 c.cm.	—	
4	100 ,,	10 c.cm.	—	10 c.cm.	Dead after 8 days.
5	100 ,,	10 c.cm.	—	10 c.cm.	Healthy.
6	100 ,,	10 c.cm.	—	10 c.cm.	Healthy.
Control	100 ,,	—	—	—	Dead after 48 hrs

CONCLUSIONS.

It has been found that in rabbits 10 c.cm. intravenous doses of a 0.5 per cent. HClO solution, even when repeated on two or three days in succession, produce no ill effects, that they may delay or prevent the onset of symptoms due to the injection of the animals with diphtheria and tetanus toxins, and with cultures of *Bacillus perfringens* and *Staphylococcus pyogenes aureus*, the rabbits recovering from the infection or remaining healthy.

Hypochlorous acid intravenously administered is therefore to be regarded as of therapeutic value; the antiseptic apparently delays the development of bacteria and destroys their toxins, thus enabling the natural resistance of the animal to assert itself.

It is suggested that this destruction of toxins may be in the nature of a protein coagulation.

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N° 2

THE AUSTRALIAN EPIDEMIC, 1914.

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In November, 1914, the Director of the Lister Institute sent me a sealed tube containing dried crusts or scabs which were stated to have been removed from patients, subjects of the Australian epidemic earlier in the year. Dr Martin requested me to investigate any ascertainable facts as to their pathological nature, and what relation, if any, they bore to vaccinia on the one hand and to variola on the other.

The clinical aspect of the epidemic has been described by Dr Armstrong[1], while certain bacteriological investigations of the disease have been reported by Drs J. Burton-Cleland and E. W. Ferguson[2].

Although the majority of the medical authorities in Australia are stated to have regarded the epidemic as one of small-pox, probably of a slight nature, doubt appears to have existed in the minds of some of the observers as to whether this was actually the case, and the present investigation is an outcome of this uncertainty.

The crusts or scabs had been forwarded to England in cold storage from Australia, and there were in all some 40 gms. of them. Throughout the time of the investigation the crusts were stored in a desiccator at a temperature of about 4° C., small quantities being removed from time to time for use as required. The material for the inoculation of animals was invariably composed as follows, one part of crusts ground up in a pestle and mortar with four times its own bulk of 50 per cent. glycerine and water, the mixture being made immediately before use. The variolar material used as a control was from a case of confluent small-pox in a man, and the specific activity of this was ascertained by inoculation on monkeys; it was strongly active throughout. The vaccine lymph used as another control was seed-lymph used for the production of lymph at these laboratories and was very active, as shown by frequent inoculations on calves.

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[1] *Proceedings of the Royal Society of Medicine, Section of Epidemiology and State Medicine,* vol. viii. No. 2, p. 1.

[2] *Ibid.* p. 19.

I.

The first series of animal experiments was made on guinea-pigs, in view of the fact that some observers had expressed the opinion that the Australian disease was a form of modified small-pox; and modification, if sufficiently extended, might give the material a vaccine character. Drs J. Burton-Cleland and E. W. Ferguson had found that the material which they employed gave typical vaccine vesicles when inoculated direct on bovines. As stated in a previous paper on vaccinia[1], if a non-immune buck guinea-pig be vaccinated on the scrotum, vaccine vesicles develop with great facility about 72 hours later. The present experiments were made in a place remote from calf vaccine work, and the technique was arranged to prevent any infection being conveyed outside. 70 buck guinea-pigs were inoculated on the scrotum with material from the Australian disease. No trace either of vesiculation or of any reaction of any kind was subsequently noted, the small incisions healing normally. 70 control pigs, inoculated at the same time and in the same manner with calf vaccine, developed in each case typical vaccine vesicles 72 hours later.

This failure to react to the crusts is no proof that the cases from which the crusts were taken were not small-pox, but it is of evidential value in tending to show that whatever modification the disease may have undergone from ordinary small-pox in its transition to "mild" small-pox (Armstrong, Burton-Cleland, Ferguson), such transition had not carried it to vaccinia. Subsequent experiments showed that the failure of the material to react was not due to loss of its own specific activity.

II.

The second series of inoculations was made on calves. The experiments on Calves 1–4 were made in the stables where routine calf vaccination is carried out, at the Lister Institute. Those on Calves 5 and 6 were made in a place remote from all such work, and the technique was arranged to obviate the possibility of any infection being conveyed away from the animals.

[1] Green. *Journal of Hygiene*, 1914.

CALF 1 (Heifer). (*a*) Was inoculated on a shaved area on the abdomen with the Australian disease. 120 hours later there were found along the lines of inoculation a series of yellow vesicles about the size of lentils; the appearance was unlike that of ordinary vaccine vesicles, being much smaller and more discrete. On the other hand their appearance was unlike that resulting from an inoculation of small-pox material direct from man to the calf, being, from my experience, too developed, even for a first inoculation positive result.

(*b*) At the same time a second area was inoculated with calf vaccine, and 120 hours later typical vaccine vesicles had developed on the site, these bearing practically no resemblance to the small yellow vesicles of the Australian disease.

CALF 2 (Bull). (*a*) Was inoculated on the shaved scrotum with the Australian disease. 120 hours later small yellow vesicles had developed as in the case of Calf 1.

(*b*) At the same time an area on the abdomen was inoculated with calf vaccine, and 120 hours later typical vaccine vesicles of first-class quality, and bearing no resemblance to the vesicles of the Australian disease, had developed here. The results on this calf resembled those on Calf 1.

CALF 3 (Heifer). (*a*) Was inoculated on a shaved area on the abdomen with vesicular contents removed from the Australian disease vesicles of Calf 1. The vesicular material had been removed at 120 hours after inoculation, and had been stored for two weeks at 4° C. 120 hours later the result on this site was *nil*.

(*b*) A second area was inoculated with similarly stored vesicular material from the Australian disease vesicles of Calf 2. 120 hours later vesicles had developed at the site, whose appearance was somewhat suggestive, but not convincing, of poor class vaccine vesicles. Passage through three subsequent calves failed to improve the quality of this vesicular material, *i.e.* to make it resemble vaccinia more closely clinically.

(*c*) A third area was inoculated with vaccine lymph and 120 hours later normal vaccine vesicles of good class had developed.

CALF 4. (*a*) A shaved area on the abdomen was inoculated with the stored material of the Australian disease vesicles of Calf 1. 120 hours later the result was *nil*.

(*b*) A second area on the abdomen was inoculated with stored material of the Australian disease vesicles from Calf 2. 120 hours later vesiculation had resulted, the vesicles resembling somewhat poor class vaccine vesicles. Passage through two further calves failed to improve the quality of the vesicles.

(*c*) A third area was inoculated with vaccine lymph and 120 hours later normal vaccine vesicles of good class had developed.

Calf 5. Was inoculated with vesicles of the Australian disease removed from Monkey No. 2 (*vide* series of monkey experiments). Vesicles resembling moderately fair quality vaccine vesicles had developed 120 hours later.

Calf 6. Was inoculated with the Australian disease vesicles removed from Monkey No. 4. 120 hours later vesicles resembling vaccine vesicles of moderately fair quality had developed.

Calf 7. (*a*) A shaved area on the abdomen was inoculated with the Australian disease. 120 hours later there was a distinct reaction, with small papules and vesicles along the lines of inoculation, the result being similar to that of Calf 1, but not quite so marked. Here again the result was not typical of vaccinia, and it was too marked for variola inoculation direct from man.

(*b*) A fortnight later a second shaved area on the abdomen was inoculated with:

(1) Variolous material direct from a case of confluent small-pox in a man. 120 hours later the results were *nil*, no reaction of any kind being noticeable;

(2) Vaccine lymph. 120 hours later typical vaccine vesicles, but not of first-class quality, had developed.

Calf 8. (*a*) A shaved area on the abdomen was inoculated with:

(1) Vaccine. 120 hours later typical vaccine vesicles had developed.

(2) Variolous material from man, passed once through a monkey, on which it had given marked typical vesiculation. 120 hours later small vesicles had developed, not typical of vaccinia (but not so well developed as those of Australian disease); they approximated more to the Australian disease vesicles than to the vaccinal vesicles however.

(*b*) A fortnight later another area on the abdomen was shaved and inoculated with Australian crust emulsion. 120 hours later there was slight vesiculation at the site about the same appearance as on Calf 5, resulting from inoculation with Australian disease.

The results of the above experiments are more easily seen in Table I.

There are thus, with regard to these calf experiments, two main questions to consider:

1. *The likeness-relation of the Australian disease to Vaccinia and to Variola.*

The most prominent fact in all the calf experiments was that the vesiculation of the Australian disease, such as it was, was not generally typical of the vaccinia vesicles on the same calf, nor, I may be allowed to remark, was it typical of any vaccine vesicles that I remember on any calf. After passage through a monkey, however, the material gave rise to vesicles undistinguishable from those of moderately fair vaccine vesicles. The general impression arrived at was that the Australian disease, both by the appearance of the vesicles, and by their time-development, possessed some vaccino-variolal relationship. Allowing that there were possibilities of such a relationship, the Australian disease would have to be classed as distinct on the one hand from vaccinia in its lesser ability to produce typical vaccinal vesiculation, though it approximated more closely after passage through a monkey; and on the other hand from variola, for without doubt it possessed a greater facility for vesiculating on a calf than did the variola in the above experiments.

In the foregoing experiments the vesicle-likeness was consistent, but it differed widely from the vesicle-likeness reported in the experiments by Drs Burton-Cleland and Ferguson. In the experiments by these observers there was a tendency for vesicles to develop very freely at any time, even in the case of repeated re-vaccinations at short intervals.

2. *The immunity-relation of the Australian disease to Vaccinia and to Variola.*

There are only two sets of data from which to draw deductions, Exps. 7 and 8 in Table I. This scarcity is due to two causes: (*a*) the comparative difficulty of obtaining calves at the present time, and maintaining them for a period necessary for the development of possible immunity and obtaining re-vaccination results; (*b*) the inferiority of the results obtained from inoculating calves with the Australian disease, and incidentally with variola—even when this had been passed through a monkey.

Taking these two sets of data for what they are worth, however, it appeared that successful vaccination with variola and vaccinia afforded no protection against subsequent inoculation with Australian disease, and the Australian disease gave no protection against vaccinia. The failure of variola to "take" on Calf 5 is of no account, as variolous material inoculated on the calf direct from man (*i.e.* not passed through a monkey) rarely gives definite vesiculation.

TABLE I.

Calf Experiments.

Calf No.	Vaccination	Result	Vaccination	Result
1.	*A*	+		
		+		
	L	+		
		+		
2.	*A*	+		
		+		
	L	+		
		+		
		+		
		+		
3.	*A* (from Calf 1)	–		
	L (from Calf 2)	+		
	L	+		
		+		
		+		
4.	*A* (from Calf 1)	–		
	A (from Calf 2)	+		
	L	+		
		+		
		+		
5.	*A*	+		
	(from Monkey No. 2)	+		
		+		
		+		
6.	*A*	+		
	(from Monkey No. 4)	+		
		+		
7.	*A*	+	*V*	–
			L	+
				+
				+
				+
8.	*L*	+		
		+		
		+		
		+		
	V (Passed once through monkey)	+	*A*	+

A = Australian disease, *L* = Calf lymph, *V* = Variola.

These two results, showing no protection, are in accord with the majority of the results of the calf experiments of Cleland and Ferguson. The accompanying Table II has been compiled for the sake of easier reference from the paper of Cleland and Ferguson[1], which should of course be consulted for full information.

It will be seen that in three instances vaccination gave a positive result following repeated successful reactions with the Australian

TABLE II.

Cleland and Ferguson's Calf Experiments.

Calf No.	2/11	4 days later	21/10	4th day?	7/11	4th day?	24/11	4th day?	11/12	4th day?	30/12	4th day?
1.	*A*	+ ?	*L*	+ + +	*A*	+	*A*	–	*A*	+	*L*	+
	16/10		7/11		24/11		11/12		30/12			
2.	*A*	+	*A*	+ +	*A*	–	*A*	+ +	*L*	+ +		
	7/11		24/11		11/12		30/12					
3.	*A*	+ + +	*A*	+	*A*	+	*L*	+				
	11/12		30/12									
4.	*A*	+ + +	*L*	–								
	24/11		17/12		9/1							
5.	*L*	+ + +	*L*	–	*A*	+						
	15/1											
6.	*A*	+ + +										
	15/11		22/1	9 days later								

Monkey Experiments.

1.	*A*	+	*L*	– ?
	22/1	9 days later		
2.	*L*	?		

A = Australian disease, *L* = calf lymph.

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[1] *Proceedings of the Royal Society of Medicine, Section of Epidemiology and State Medicine*, Vol. VIII. No. 2, p. 1.

disease, and only one case a negative. In one case, Calf 5, the authors obtained a positive result of Australian inoculation following successful vaccination, *i.e.* complete protection was afforded only in 25 per cent. of the cases. In view of this fact it is difficult to understand Cleland and Ferguson's claim—"This inoculated disease and vaccinia are mutually more or less completely protective against each other provided sufficient area is inoculated." It would seem, indeed, that the number of Cleland and Ferguson's experiments is far too small to permit of the attempt to select one of the five (the sixth was not, of course, a cross immunity experiment at all) as establishing a law. The only deduction that it would seem safe to draw is the obvious one before mentioned, that 75 per cent. of the cases of inoculated Australian disease failed to protect against subsequent vaccination.

As before stated, the results of the experiments on calves in the present series of experiments were not such as to allow one to place much reliance on them in any way, and for this reason the series of experiments was not extended, extension preferably being made with monkey experiments, which gave very much better inoculation results.

III.

The third series of experiments was made on rhoesus and bonnet monkeys. The work was done in a place remote from all calf vaccine work, in order that no question of any accidental inoculation could arise, and under conditions of strict isolation to prevent the possibility of any infection being conveyed away from the monkeys. Each animal during the experimental period was kept in a separate cage by itself. Each animal was inoculated on an area of shaved skin over the scapulae, in order to reduce to a minimum the prospect of the results being spoilt by scratching. Each shaved area was about 6 square cm., and two linear incisions were made close together, allowing sufficient room for subsequent inoculations.

MONKEY 1. *Inoculation.* 1. xii. 14. Vaccinia.

Result. Typical vaccination vesicles of rather poor quality.

Inoculation. 12. i. 15. Australian disease.

Result. Definite vesiculation undistinguishable from results of calf vaccine of poor quality.

Conclusion. Here a successful vaccination with vaccinia afforded no protection against the subsequent inoculation of Australian disease.

MONKEY 2. *Inoculation.* 1. xii. 14. Australian disease.

Result. Well developed vesicles, resembling typical vaccine vesicles of good quality. Subsequent inoculation on Calf 5 gave vesiculation resembling that of calf vaccine.

Inoculation. 12. i. 15. Vaccinia.

Result. 19. i. 15. Full well developed vesicles of "good" quality.

Inoculation. 9. ii. 15. On one area vaccinia, and on another Australian disease.

Result. 16. ii. 15. *Nil* in each case.

Conclusion. This experiment suggested that while Australian disease failed to protect against vaccinia, the original inoculations of vaccine and Australian disease afforded protection against subsequent inoculation of the virus of these diseases—lymph against lymph and Australian disease against Australian disease.

MONKEY 3. *Inoculation.* 1. xii. 14. Australian disease.

Result. 8. xii. 14. Definite vesiculation, but tendency for discrete vesiculation to form along the lines of incision and for vesicles to be irregular.

Inoculation. 12. i. 15. Vaccinia.

Result. 19. i. 15. Typical vesiculation with tendency to irregularity.

Conclusion. There was manifestly no protection afforded here by the Australian disease against vaccinia.

MONKEY 4. *Inoculation.* 8. xii. 14. Australian disease.

Result. 15. xii. 14. Well developed vesicles undistinguishable from vaccine vesicles of good quality. This vesicular material inoculated on Calf 6 gave vesicles resembling vaccine vesicles of fair quality.

Inoculation. 12. i. 15. Vaccinia.

Result. 19. i. 15. Typical vesiculation of good quality.

Inoculation. 9. ii. 15. Australian disease on one area, vaccinia on another.

Result. 16. ii. 15. *Nil* in each case.

Conclusion. The experiment would appear to indicate that Australian disease failed to protect against subsequent vaccinia, but that these two diseases protected against a further subsequent double inoculation on separate areas of the same diseases.

MONKEY 5. *Inoculation.* 9. ii. 15. Australian disease.

Result. 16. ii. 15. Rather poor vesiculation.

Inoculation. 2. iii. 15. Variola.

Result. 9. iii. 15. *Nil.*

Conclusion. Australian disease possibly protected against variola.

Monkey 6. *Inoculation.* 9. ii. 15. Australian disease.

Result. 16. ii. 15. Good vesiculation.

Inoculation. 2. iii. 15. Vaccinia on one area, variola on the other.

Result. 9. iii. 15. Vaccinia doubtful, variola good vesiculation.

Conclusion. Australian disease did not protect against variola, and probably not against vaccinia.

Monkey 7. *Inoculation.* 9. ii. 15. Vaccinia.

Result. 16. ii. 15. Good vesiculation.

Inoculation. 2. iii. 15. Vaccinia on one area, variola on another.

Result. 9. iii. 15. Vaccinia *nil*, variola *nil.*

Inoculation. 23. iii. 15. Australian disease.

Result. 30. iii. 15. Slight papulation.

Conclusion. Vaccinia protected against vaccinia and variola, but not against Australian disease.

Monkey 8. Died before any cross immunisation could be attempted.

Monkey 9. Died before any cross immunisation could be attempted.

Monkey 10. *Inoculation.* 9. ii. 15. Variola.

Result. 16. ii. 15. Fairly good vesiculation.

Inoculation. 2. iii. 15. Australian disease on one area, vaccinia on another.

Result. 9. iii. 15. Australian disease good vesiculation, vaccinia *nil.*

Conclusion. Variola protected against vaccinia, but not against Australian disease.

Monkey 11. *Inoculation.* 16. ii. 15. Variola.

Result. 23. ii. 15. Fair vesiculation.

Inoculation. 2. iii. 15. Australian disease on one area, vaccinia on another.

Result. 9. iii. 15. Australian disease definite but slight vesiculation, vaccinia *nil.*

Conclusion. Variola protected against vaccinia, but not against Australian disease, or only to a very moderate degree.

Monkey 12. *Inoculation.* 23. ii. 15. Mixed aerobic and anaerobic growth, cocci in broth, cultivated from Australian disease crusts.

Result. 2. iii. 15. *Nil.*

Inoculation. 2. iii. 15. Australian disease.

Result. 9. iii. 15. Definite vesiculation.

Inoculation. 23. iii. 15. Vaccinia on one area, variola on another.

Result. 30. iii. 15. Vaccinia fair vesiculation, variola *nil.*

Conclusion. (1) Cocci cultivated from crusts not pathogenic. (2) Australian disease did not protect against vaccinia, but may have protected against variola.

MONKEY 13. *Inoculation.* 2. iii. 15. Vaccinia on one area, variola on another.

Result. 9. iii. 15. Vaccinia fair vesiculation, variola strongly marked vesiculation.

Inoculation. 23. iii. 15. Australian disease.

Result. 30. iii. 15. Fair vesiculation.

Conclusion. Vaccinia and variola have not protected against Australian disease.

MONKEY 14. *Inoculation.* 2. iii. 15. Australian disease on one area, variola on another.

Result. 9. iii. 15. Australian disease fair vesiculation, variola poor vesiculation.

Inoculation. 23. iii. 15. Vaccinia.

Result. 30. iii. 15. Good vesiculation.

Conclusion. Australian disease does not protect against vaccinia, but neither did variola protect against vaccinia; it must be noted that the variola gave poor result.

MONKEY 15. *Inoculation.* 10. iii. 15. Variola.

Result. 17. iii. 15. Good vesiculation.

Inoculation. 23. iii. 15. Australian disease on one area, vaccinia on another.

Result. 30. iii. 15. Australian disease fair vesiculation, vaccinia *nil.*

Conclusion. Variola protected against vaccinia, but not against Australian disease.

MONKEY 16. *Inoculation.* 10. iii. 15. Variola.

Result. 17. iii. 15. Very marked vesiculation.

Inoculation. 23. iii. 15. Australian disease on one area, vaccinia on another.

Result. 30. iii. 15. Australian disease *nil,* vaccinia poor vesiculation.

Conclusion. Variola protected against Australian disease, and not against vaccinia, or only very slightly.

MONKEY 17. *Inoculation.* 10. iii. 15. Variola.

Result. 17. iii. 15. Rather poor vesiculation.

Inoculation. 23. iii. 15. Australian disease on one area, vaccinia on another.

Result. 30. iii. 15. Australian disease *nil*, vaccinia poor vesiculation.

Conclusion. Variola protected against Australian disease, but not against vaccinia, or only partially.

MONKEY 18. *Inoculation.* 10. iii. 15. Australian disease.

Result. 17. iii. 15. Vesiculation.

Inoculation. 23. iii. 15. Vaccine on one area, variola on another.

Result. 30. iii. 15. Vaccine first-class vesicles, variola fairly good vesicles.

Conclusion. Australian disease gave no protection against vaccine or variola.

MONKEY 19. *Inoculation.* 10. iii. 15. Australian disease.

Result. 17. iii. 15. Vesiculation fair.

Inoculation. 23. iii. 15. Vaccinia on one area, variola on another.

Result. 30. iii. 15. Poor vesiculation in each case.

Conclusion. Australian disease afforded no protection against vaccinia or variola, or only partial protection.

MONKEY 20. *Inoculation.* 10. iii. 15. Australian disease.

Result. 17. iii. 15. Vesiculation.

Inoculation. 23. iii. 15. Vaccinia on one area, variola on another.

Result. 30. iii. 15. Vaccinia good vesicles, variola very good.

Conclusion. Australian disease had afforded no protection against vaccinia or variola.

MONKEY 21. *Inoculation.* 10. iii. 15. Vaccinia.

Result. 17. iii. 15. Fair vesiculation.

Inoculation. 23. iii. 15. Australian disease on one area, variola on another.

Result. 30. iii. 15. Australian vesiculation good, variola *nil*.

Conclusion. Vaccinia afforded protection against variola, but not against Australian disease.

MONKEY 22. *Inoculation.* 10. iii. 15. Vaccinia.

Result. 17. iii. 15. Fair vesiculation.

Inoculation. 23. iii. 15. Australian disease on one area, variola on another.

Result. 30. iii. 15. Australian disease *nil*, variola *nil*.

Conclusion. Vaccinia protected against variola, and may have protected against Australian disease; at any rate the Australian disease failed to develop after previous successful vaccination.

The foregoing results have been condensed and tabulated in Table III.

TABLE III.

Monkey No.	Inoculation and Date	Result	Inoculation and Date	Result	Inoculation and Date	Result
1.	1/12/14 *L*	8/12/14 +	12/1/15 *A*	19/1/15 + Died		
2.	1/12/14 *A*	8/12/14 + + +	12/1/15 *L*	19/1/15 + + +	9/2/15 *L* ⋮ *A*	16/2/15 – ⋮ – Died
3.	1/12/14 *A*	8/12/14 + +	12/1/15 *L*	19/1/15 + + Died		
4.	8/12/14 *A*	15/12/14 + + +	12/1/15 *L*	19/1/15 + + +	9/2/15 *L* ⋮ *A*	16/2/15 – ⋮ –
5.	9/2/15 *A*	16/2/15 +	2/3/15 *V*	9/3/15 –		
6.	9/2/15 *A*	16/2/15 + +	2/3/15 *V* ⋮ *L*	9/3/15 + ⋮ ?		
7.	9/2/15 *L*	16/2/15 + + +	2/3/15 *V* ⋮ *L*	9/3/15 – ⋮ –	23/3/15 *A*	30/3/15 +
8.	9/2/15 *L*	16/2/15 + Died				
9.	9/2/15 *V*	16/2/15 + Died				
10.	9/2/15 *V*	16/2/15 + +	2/3/15 *A* ⋮ *L*	9/3/15 + ⋮ –		
11.	16/2/15 *V*	23/2/15 +	2/3/15 *A* ⋮ *L*	9/3/15 + ⋮ –		
12.	23/2/15 Cocci from *A*	2/3/15 –	2/3/15 *A*	9/3/15 +	23/3/15 *L* ⋮ *V*	30/3/15 + ⋮ –
13.	2/3/15 *L* ⋮ *V*	9/3/15 + ⋮ +	23/3/15 *A*	30/3/15 +		
14.	2/3/15 *A* ⋮ *V*	9/3/15 + ⋮ +	23/3/15 *L*	30/3/15 + + +		
15.	10/3/15 *V*	17/3/15 + +	23/3/15 *A* ⋮ *L*	30/3/15 + ⋮ –		

TABLE III—(*continued*).

Monkey No.	Inoculation and Date	Result	Inoculation and Date	Result
	10/3/15	17/3/15	23/3/15	30/3/15
16.	*V*	+ + + +	*A* : *L*	– : +
	10/3/15	17/3/15	23/3/15	30/3/15
17	*V*	+	*A* : *L*	– : +
	10/3/15	17/3/15	23/3/15	30/3/15
18.	*A*	+	*L* : *V*	+ : + : + + : + + :
	10/3/15	17/3/15	23/3/15	30/3/15
19.	*A*	+	*L* : *V*	+ : +
	10/3/15	17/3/15	23/3/15	30/3/15
20.	*A*	+	*L* : *V*	: + + : + + : + : +
	10/3/15	17/3/15	23/3/15	30/3/15
21.	*L*	+	*A* : *V*	+ : –
	10/3/15	17/3/15	23/3/15	30/3/15
22.	*L*	+	*A* : *V*	– : –

V=variola, *L*=calf lymph, *A*=Australian disease.

As in the case of the calf experiments it will be convenient to consider this series of monkey experiments in two main aspects.

1. *The likeness-relation of the Australian disease to Vaccinia and to Variola.*

In this series (as has been previously remarked in the general statement) there is little or none of the ambiguity attaching to the question that appeared in the case of the calf experiments. Without exception the Australian disease vesicles resembled typical vaccine vesicles so closely that it was impossible to distinguish one condition from the other. In time-relation too the two appeared identical, there was indeed no apparent clinical distinction.

2. *The immunity-relation of the Australian disease to Vaccinia and to Variola.*

For the clearer consideration of this problem the experiments have been divided into sub-series in the following four tables (Tables IV, V, VI and VII).

In the first sub-series (*Table IV*). Those experiments have been collected in which monkeys were in the first place inoculated with

TABLE IV.

(*Sub-series* 1.)

Monkey No.	Inoculation and Date	Result	Inoculation and Date	Result	Inoculation and Date	Result
2.	1/12/14 *A*	8/12/14 + + +	12/1/15 *L*	19/1/15 + + +	9/2/15 *L* : *A*	16/2/15 – : – Died
3.	1/12/14 *A*	8/12/14 + +	12/1/15 *L*	9/2/15 + + Died		
4.	8/12/14 *A*	15/12/14 + + +	12/1/15 *L*	19/1/15 + + +	9/2/15 *L* : *A*	16/2/15 – : –
5.	9/2/15 *A*	16/2/15 +	2/3/15 *V*	9/3/15 –		
6.	9/2/15 *A*	16/2/15 + +	2/3/15 *L* : *V*	9/3/15 + : +		
18.	10/3/15 *A*	17/3/15 +	23/3/15 *L* : *V*	30/3/15 + : + : + + : + + :		
19.	10/3/15 *A*	17/3/15 +	23/3/15 *L* : *V*	30/3/15 + : +		
20.	10/3/15 *A*	17/3/15 +	23/3/15 *L* : *V*	30/3/15 : + + : + + : + : +		

V = variola, *L* = calf lymph, *A* = Australian disease.

TABLE V.

(*Sub-series* 2.)

Monkey No.	Inoculation and Date	Result	Inoculation and Date	Result	Inoculation and Date	Result
1.	1/12/14 *L*	8/12/14 +	12/1/15 *A*	19/1/15 + Died		
7.	9/2/15 *L*	16/2/15 + + + +	2/3/15 *L* : *V*	9/3/15 – : –	23/3/15 *A*	30/3/15 +
21.	10/3/15 *L*	17/3/15 + +	23/3/15 *A* : *V*	30/3/15 + : –		
22.	10/3/15 *L*	17/3/15 + +	23/3/15 *A* : *V*	30/3/15 – : –		

A = Australian disease, *V* = variola, *L* = calf lymph.

TABLE VI.

(*Sub-series* 3.)

Monkey No.	Inoculation and Date	Result	Inoculation and Date	Result
10.	9/2/15 *V*	16/2/15 + +	2/3/15 *A* : *L*	9/3/15 + : –
11.	16/2/15 *V*	23/2/15 + +	2/3/15 *A* : *L*	9/3/15 + : –
15.	10/3/15 *V*	17/3/15 + +	23/3/15 *A* : *L*	30/3/15 + : –
16	10/3/15 *V*	17/3/15 + + + +	23/3/15 *A* : *L*	30/3/15 – : +
17.	10/3/15 *V*	17/3/15 +	23/3/15 *A* : *L*	30/3/15 – : +

A = Australian disease, *V* = variola, *L* = calf lymph.

TABLE VII.

(*Sub-series* 4.)

Monkey No.	Inoculation and Date	Result	Inoculation and Date	Result
12.	2/3/15 *A* : *L*	9/3/15 + : + : +	23/3/15 *V*	30/3/15 –
13.	2/3/15 *L* : *V*	9/3/15 + : + + : + : +	23/3/15 *A*	30/3/15 + +
14.	2/3/15 *A* : *V*	9/3/15 + : + : +	23/3/15 *L*	30/3/15 + + +

A = Australian disease, *V* = variola, *L* = calf lymph.

Australian disease and subsequently with vaccinia and variola—one after the other, or simultaneously.

In the second sub-series (*Table V*). Those experiments have been collected in which monkeys were in the first place inoculated with vaccinia, and subsequently with Australian disease and variola—one after the other, or simultaneously.

In the third sub-series (*Table VI*). Those experiments have been collected in which monkeys were in the first place inoculated with variola, and subsequently with Australian disease and vaccinia—in every case simultaneously.

In the fourth sub-series (*Table VII*). Those experiments, only three in number, have been collected which do not conform with those of the previous groups, and consist of initial double inoculations, with subsequent cross immunisation inoculations, as shown in the Table.

In sub-series 1. The eight initial inoculations of Australian disease, as shown in Table IV, were all successful in yielding vesicles, and some of the vesicles were of noticeably good quality, and well developed. Of these there were three cases of subsequent inoculation with vaccinia alone which all yielded vesicles, one case of subsequent inoculation with variola, which gave no reaction, and four cases of subsequent inoculation with vaccinia and variola simultaneously, which all gave definite typical vesiculation. In the whole eight cases therefore of this sub-series there was only one case (Monkey 5) to suggest that Australian disease afforded protection against vaccinia or variola. On the other hand the remaining seven cases indicate that no protection whatever had been afforded by Australian disease against subsequent vaccinia or variola. It only seems possible therefore to state that the percentage of immunity afforded by Australian disease in this subsection was *nil* or very small.

That these animals were capable of developing immunity is shown in the cases of Monkeys 2 and 4, where re-inoculations of Australian disease and vaccinia respectively gave negative results.

In sub-series 2 (*Table V*). Consisting of four experiments, all initial inoculations with vaccinia gave typical vesiculation. All four animals were subsequently inoculated with Australian disease, and in three instances this was followed by vesiculation typical of vaccinia vesicles; the remaining one failed to react—a result of 75 per cent. against, and 25 per cent. for protection.

In sub-series 3 (*Table VI*). The five monkeys were all primarily inoculated with variola and gave vesiculation undistinguishable from the vesicles of vaccinia and Australian disease. Each case was subsequently inoculated with Australian disease and vaccinia simultaneously; in three cases the Australian inoculation was followed by typical vesiculation and in two cases by no reaction, indicative of conferred immunity in 40 per cent. and absence of conferred immunity in 60 per cent. If we take the cases of sub-series 2 and 3 together, vaccinia or variola may have conferred immunity in 33·3 per cent. cases, and did not confer it in 66·6 per cent. It must be noted however, that in sub-series 3 the possible protection afforded against Australian disease occurred in the same percentage as the protection afforded by variola against vaccinia.

In sub-series 4 (*Table VII*). In Monkey No. 12, Australian disease or vaccinia protected against subsequent inoculations with variola; in Monkey No. 13, vaccinia and variola failed to protect against Australian disease; and in Monkey No. 14 Australian disease and variola failed to protect against vaccinia.

Taking the four sub-series as a whole, evidence as to the ability of Australian disease to protect against vaccinia or variola was small or absent, but vaccinia or variola possibly protected against Australian disease in 30 per cent. of the cases; vaccinia protected against variola in 100 per cent. of the cases, and variola protected against vaccinia in 50 per cent. of the cases.

In comparing the foregoing percentage of vaccinia and variola protection one against the other, it is important to remember that the variola was inoculated experimentally, *i.e.* the infective material gained both a more intimate contact with the tissues than would be the case in usual variola infection, and was applied in much larger doses. The same point must be noted in connection with the Australian disease, and the fact that protection was afforded against variola and not against Australian disease, has the more significance, inasmuch as it occurred under this more stringent application of infection.

It may be noted in the foregoing text that when vaccinia or variola has failed to develop after previous successful inoculation with Australian disease the fact has been stated as such; but when variola has failed to develop after previous successful vaccination it is definitely assumed that this is in consequence of the vaccination. This variation of expression is owing to the circumstance that it is an accepted fact that vaccinia protected against variola, but it is not yet an accepted fact that Australian disease protects against vaccinia or variola.

Briefly stated, the conclusions to be drawn from the foregoing experiments as a whole are that, clinically, the Australian disease:

1. Bears no likeness-relation to vaccinia in guinea-pigs, possibly some slight likeness-relation to vaccinia in calves, also to variola in calves when the variola has been passed through a monkey; this relationship would seem to be an intermediate one, between vaccinia on one hand and variola on the other. In monkeys Australian disease is practically undistinguishable from vaccinia or variola.

2. From the monkey experiments, which afford the only evidence of value in this respect, the Australian disease bears a slighter immunity relationship to vaccinia and to variola than either vaccinia or variola bear to each other, and this in spite of the fact that the clinica

relationship of the Australian disease shows certain definite signs of being intermediate between vaccinia on the one hand and variola on the other.

SUMMARY.

(1) Inoculation of pathologically active crusts on guinea-pigs caused no reaction, and in this respect a marked difference was demonstrated clinically between the disease of which the crusts were a product, and vaccinia.

(2) On calves the disease tended to show some vaccino-variolal relationship, from the appearance of its vesicles, but it appeared to be distinct on the one hand from vaccinia, and from variola on the other.

(3) Calf experiments, two only in number, suggested that vaccinia and variola failed to protect against Australian disease, and that Australian disease failed to protect against vaccinia or variola.

(4) On monkeys the vesicles of Australian disease were undistinguishable in appearance from those of vaccinia or variola, but the evidence for immunity relationship between Australian disease and vaccinia and variola was of the slightest; Australian disease affording no protection against vaccinia or variola, vaccinia possibly protecting against Australian disease in 25 per cent. of the cases, and variola possibly protecting against Australian disease in 40 per cent. of the cases. Vaccinia protected against variola in 100 per cent. of the cases, and variola against vaccinia in 50 per cent. of the cases.

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The Outbreak of Cerebrospinal Fever at Salisbury in 1914-15.

By M. GREENWOOD, Jun., Captain R.A.M.C.(T.F.).[1]

THE following observations summarize the statistical section of a report on the Salisbury outbreak which I was instructed to prepare in collaboration with Dr. W. J. Penfold, of the Lister Institute. Dr. Penfold's absence from England makes it impossible to complete the report, but, as some of the facts may be of interest in connexion with Dr. Hamer's paper, it seemed desirable to publish these notes. It is to be hoped that Dr. Penfold will ultimately discuss the whole subject.

At an early stage of the epidemic which attacked the civilian population of Salisbury in the winter of 1914-15, Dr. Penfold was instructed by the Governing Body of the Lister Institute to visit the city and collaborate with the local authorities. A laboratory was equipped and preparations made to supply vaccine for the prophylactic inoculation of all who desired to avail themselves of the process. A polyvalent vaccine, embracing in most cases six distinct strains of meningococci, was manufactured, the strains being all derived from Salisbury cases. Adults normally received a first dose of 250 to 300 millions, followed a week later by 1,000 millions. No untoward results of inoculation were observed and the local reactions were, in the great majority of cases, slight. In all, over 4,000 persons were inoculated, and particulars of most of these inoculations are furnished below.

At the outset, certain general features of the epidemic are worthy of notice. In Tables I and II are recorded the cases of cerebrospinal fever notified among the civilian population of the city, and similar particulars respecting the troops within the Salisbury Plain District. The diagram, in which the monthly returns are reduced to percentages, enables the two records to be more easily compared (*see* p. 3).

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[1] From the Lister Institute of Preventive Medicine.

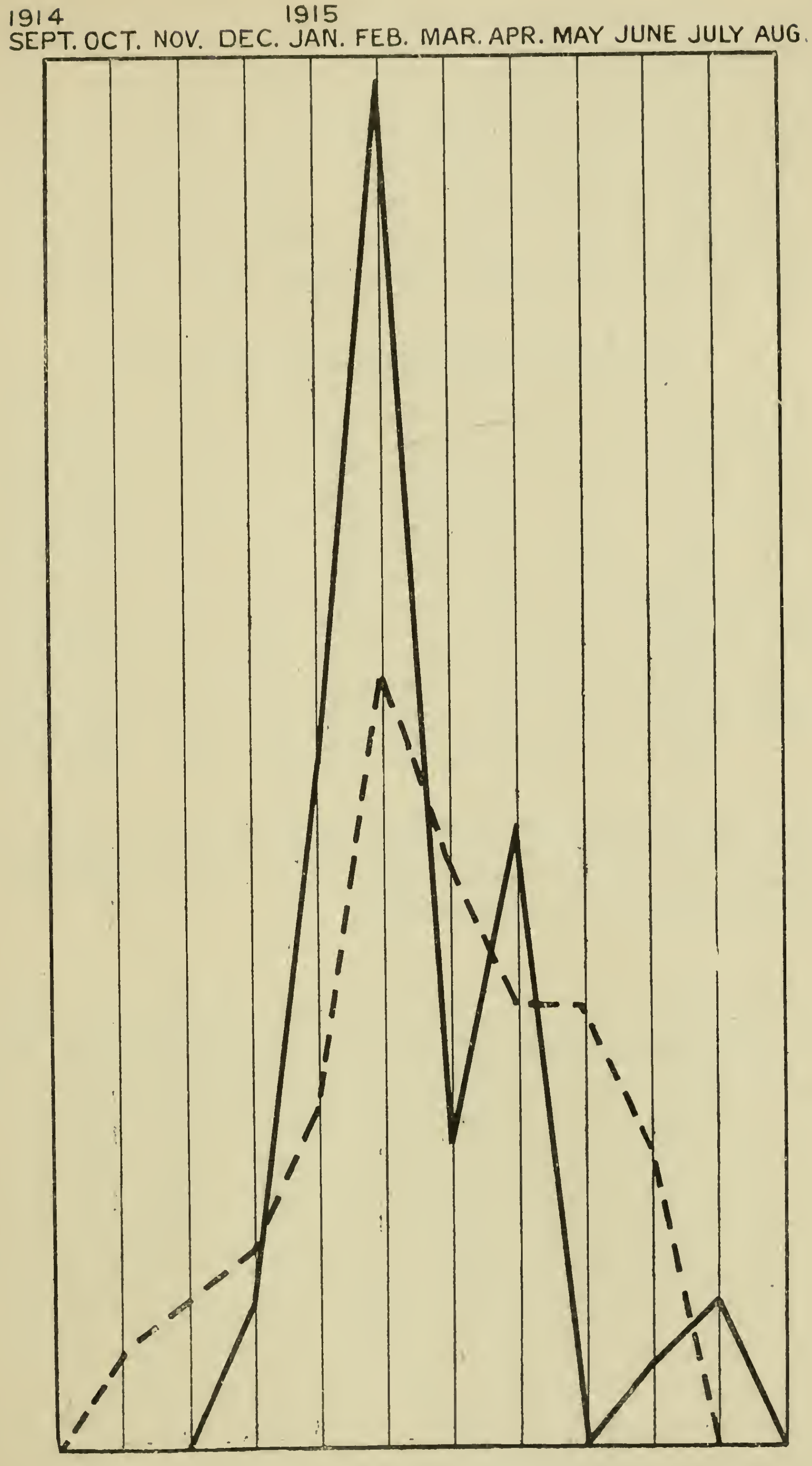
TABLE I. MONTHLY DISTRIBUTION OF CASES OF CEREBROSPINAL FEVER IN SALISBURY.

Month		Number of cases
December,	1914	2
January,	1915	9
February,	,,	18
March,	,,	4
April,	,,	8
May,	,,	—
June,	,,	1
July,	,,	2
		44

TABLE II.—MONTHLY DISTRIBUTION OF CASES AMONG THE TROOPS IN SALISBURY PLAIN DISTRICT.

Month		Number of cases
October,	1914	2
November,	,,	3
December,	,,	4
January,	1915	7
February,	,,	16
March,	,,	12
April,	,,	9
May,	,,	9
June,	,,	6
		68

It will be seen that the course of events was not the same in the two outbreaks. Both show a maximum in February, but the city epidemic declined more rapidly thereafter than did that among the troops. As the absolute numbers are small, this difference might perhaps be regarded as unessential. The point can, however, be tested by a method devised by Pearson [4], and the application of his method shows that if we regard each month's cases as a separate group, the odds against the two records having arisen by sampling from a common population are about sixty-three to one. If the groups are widened, the October, November, December and January cases being classed together, and those for May, June and July also grouped together, the odds fall to some twelve to one, but are still appreciable. It is, therefore, hardly safe to regard the difference between the two records as a mere error of sampling. The populations at risk were exposed to almost identical meteorological conditions, but, of course, differed in age- and sex-constitution. This difference *may* influence the form of the epidemic curve, but there is no obvious reason why it should do so although we should expect it to modify the *general* severity of the outbreak. Another possible explanation must certainly be borne in mind.



Proportional incidence of cerebrospinal fever in different months. Full line gives civilian cases, broken line military cases.

Inoculation was introduced in February, and Dr. Penfold, together with the local authorities, displayed so much energy that by the first week of March something like 10 per cent. of the population had been inoculated. In other words, the appearance of a large proportion of inoculated persons coincides with the decline of the epidemic at a faster rate than prevailed in the case of the uninoculated troops of the surrounding districts. No careful reasoner would assign decisive importance to this coincidence, but it deserves to be recorded.

TABLE III.—WEEKLY INOCULATIONS IN SALISBURY.*

Period		Inoculations performed		Totals	
February	15-21	22	(22)	22	(22)
,,	22-28	800	(846)	822	(868)
March	1-7	1,691	(1,755)	2,513	(2,623)
,,	8-14	706	(729)	3,219	(3.352)
,,	15-21	140	(142)	3,359	(3,494)
,,	22-28	43	(41)	3,402	(3,538)
March 29—April 4		22	(22)	3,424	(3,560)
April	5-11	16	(16)	3,440	(3,576)
,,	12-18	46	(46)	3,486	(3,622)
,,	19 25	101	(101)	3,587	(3,723)
April 26—May 2		86	(87)	3,673	(3,810)
,,	3-9	26	(26)	3,699	(3,836)
,,	10-16	4	(4)	3,703	(3,840)

* The figures in parentheses include additions received after the analysis had been completed. To the total of 3,840 can be added 334, the number of inoculations known to have been performed, the records of which do not give dates. This grand total of 4,174 is still incomplete.

Let us now turn to the details of the inoculations. Table III contains the data available at the time the analysis was prepared (February, 1916). Since then 393 further names have been received. Of these, 137 could be tabulated by date of first inoculation. As, however, the additions do not finally complete the total, and their incorporation in the tables would not sensibly affect the argument, the analysis has not been repeated. From the figures in the table, and an estimate of the numbers inoculated and not there recorded, we can safely conclude that by March 7 between 2,000 and 3,000 persons had received at least one dose of vaccine (the tabulation is by date of *first* inoculation). We have unfortunately no accurate knowledge of the total population of Salisbury at this time, owing to the disturbance produced by the War in a city which is the geographical centre in a ring of camps and the Headquarters of an Army Command.

It can, however, be surmised that the number of residents was greater than at the time of the census in 1911. Apart from natural increase, losses due to men joining the colours were probably more than counterbalanced by an influx of civilian labourers and other persons employed by the military authorities. The effective population is likely to have been between 22,000 and 24,000. But this fact, coupled with the circumstance that after March 7 only fifteen cases of cerebrospinal fever occurred, makes it quite hopeless to expect to learn anything of value from a comparison of the incidence of the disease upon inoculated and uninoculated populations as wholes. The possibility of errors of sampling deprives such a comparison of any force.

The truth of this contention will now be demonstrated. Mr. Udny Yule and myself have pointed out that there are objections to the use of Pearson's well-known "Goodness of Fit Test" in cases of the present kind [3], but we recognized its value in rough practice and it may well be used here. If then we take the uninoculated population to have been, in round numbers, 20,000, and the inoculated 2,500, and if no cases of disease occurred among the inoculated, how many cases should we require to find among the uninoculated to make it probable (by Pearson's test) that the two groups are differentiated? We have the following table:—

	Not attacked	Attacked	Totals
Inoculated	2,500 ...	0 ...	2,500
Not inoculated	$20{,}000 - x$ ...	x ...	20,000
Totals ...	$22{,}500 - x$ ..	x ...	22,500

What value of x will give us χ^2 equal to, say, 8 (which corresponds to $P = 0{\cdot}046$, or odds of 954 to 46 against the difference being a chance event)? Solving for x on this assumption, we find that it is approximately equal to 64. That is to say, we should require to find more cases among the uninoculated than were recorded during the whole epidemic season in order to invest the absence of disease among the inoculated with statistical significance. Let us pass then to the special age-groups which are, according to previous experience, peculiarly liable to attack. These groups will consist of persons up to 5 years of age, 5 but under 10, 10 but under 15. The numbers inoculated up to and including March 7 are shown in Table IV, so far as they are known, and none of these persons was attacked by cerebrospinal fever. The city population under the age of 15 is less likely to have been seriously disturbed by war conditions than that at adult ages, and may be estimated as 6,023. Seven cases of cerebrospinal fever were notified as having occurred among persons of the ages in question during the period from March 7. Consequently we have this state of affairs: 983 inoculated with no cases, 5,040 uninoculated with seven cases. Might this arrangement be the result of random sampling? The answer is that, whether the four-fold test above mentioned, or another and possibly better test be employed, the odds against this being a mere chance happening are small (the former test actually makes the arrangement more likely than not—i.e., if the samples really came from a common population we should, in the long run, get as great a divergence as observed, or a greater, in more than 50 per cent. of trials). This conclusion is not sensibly affected even if we assume that another 200 of the unrecorded but inoculated fell within the age-groups.

TABLE IV.—NUMBERS INOCULATED WITHIN CERTAIN AGE GROUPS UP TO MARCH 7, 1915.

Age group	Number
—5	74
5—10	364
10—15	545
	983

[This table does not include the later returns, but only a few of the later returns come within the assigned limits.]

It is important to realize exactly what has been proved. We have *not* proved that the inoculations were useless. *Prima facie* their efficacy was complete, for no inoculated person contracted the disease; but there were not sufficient cases of the inoculated for us to be able to assert with confidence that the favourable result was more than a chance happening. It may be added that this method of computing and comparing incidence rates in terms of the whole population of a town is, in the writer's opinion, of very little value. It can only be appropriate when we know, or may reasonably assume, that all the persons composing the population were equally exposed to risk. This assumption is very rarely justifiable in the case of a disease which occurs in epidemic form, and not usually appropriate even in the instance of an endemic disease. Thus, in the former case, inhabitants of the house or street which furnished the initial cases, whether these be importations or arising *de novo*, are peculiarly exposed to risk, while, in the latter, differences of social class may be of moment, not to speak of other factors.

I desire here to call attention to an epidemiological problem which is not always studied so closely as it deserves—viz., the respective shares of infection and unfavourable environment in the production of multiple cases of disease. We all recognize that the mere occurrence of a series of cases in a house is no proof of contagion, and can cite many examples from the history of, for instance, enteric fever, but we are nevertheless a little prone to attribute such sequences to contagion or at least personal infection, when we have to deal with, epidemiologically speaking, less familiar diseases. In general terms we may say that a distribution of cases in which infection plays no part will be some function of constant probabilities of falling a victim, while if personal infection is involved the probabilities themselves will be functions of the numbers of initial cases. But, apart from the difficulties of exhaustively enumerating the hypotheses based upon an assumed constancy of risks the distinction is still too absolute. The risk might *appear* to be a function of the number already affected (as, for instance, deaths in a bombardment when the number of guns concentrated upon a certain trench was steadily increased), although the cases themselves had nothing to do with spreading the disease. I think it is possible to throw some light on this difficulty by mathematical methods, and hope to discuss the problem in a future paper; it is only mentioned in this note to guard against the risk of seeming to attach too much importance to the inquiry next described.

The evidence adduced by Chalmers, Farrar, and others, reveals a tendency on the part of cerebrospinal fever to show itself as aggregations of cases at foci with little tendency towards general dissemination. Thirty years ago Wolff commented on the fact that the 180 cases of cerebrospinal fever recorded in Hamburg between 1880-85 occurred in 131 streets, about one-eighth of all the streets in Hamburg, and not containing one-eighth of the total population. Chalmers and others have noted the apparent relation between the occurrence of multiple cases and the existence of bad ventilation (not necessarily dirt) in the dwellings. These results point to the establishment of danger zones. But the only evidence we can usually have of the existence of such danger zones is the occurrence of a case or cases. Perhaps this case, arising it may be fortuitously, creates the danger, perhaps it is merely the objective sign of the existence of other conditions, such as imperfect ventilation, likely to react upon others. Without trying to solve this problem, the essential difficulties of which have been mentioned above, we seem justified in making the point that the associates of a patient are subject to a greater than average risk of themselves taking the disease. The rarity of military contact infections does not invalidate the conclusion. It is true that the degree of overcrowding in sleeping quarters is usually much higher than within even the poorest class of civilian dwellings, but the closeness of association, day in, day out, between the members of a civilian family is considerably greater since so large a proportion of time is spent by soldiers in the open air, and the civilian population includes young children.

For these reasons I thought it well to inquire into the fate of the contacts of the Salisbury cases. It was found that of 240 known contacts 190 had been vaccinated, and fifty had not been vaccinated. No cases of cerebrospinal fever occurred among the former, among the latter there were four cases. In arriving at the number of cases among contacts only those developing after close and direct association with a previous case were reckoned. The four are made up of Mrs. D. and Corporal R., house contacts of E. D., and of W. S. and T. R., workmates of a patient. The evidence connecting at least two other cases with a previous one is strong, but not, in my opinion, conclusive. Tabulating these results, we have:—

	Inoculated	Not Inoculated	Totals
Not affected	190	46	236
Affected	0	4	4
Totals	190	50	240

Which leads to $\chi^2 = 15·458$ and $P = 0·0015$, or odds of 665·6 to 1 against the event. If we apply Bayes's theorem, we find that after 190 trials with no successes the odds are 594 to 1 against getting four or more successes in fifty trials. Alternatively, the probability of getting a sample of 190 without a success after fifty trials with four successes is 0·00036, and the mean of the two chances is 0·001. Either method leads to the conclusion that the observed difference between the fortunes of the uninoculated and the inoculated contacts is hardly likely to be an error of sampling.

Let us now criticize the result. The comparison is not vitiated by any unfavourable age constitution of the uninoculated contacts. On the contrary, the inoculated subjects comprised a larger proportion of children and young adults. Neither is it rendered illegitimate by any evidence of closer association between the uninoculated contacts and the patients than obtained among the inoculated. So far, so good; but two other objections cannot be so easily met.

The first is, that two out of the four cases, and everything turns upon but four cases, occurred at the end of January when the epidemic was at its height. At this stage few had been inoculated, so that most of the contacts then were uninoculated. If the disease is infectious, and if its infectivity is a function of time, that is to say, if cases arising towards the end of an epidemic are less likely to produce secondary cases than those occurring at the beginning or middle of the epidemic, the comparison instituted is unfair. I admit the hypothetical nature of the objection, but in our existing state of ignorance of the epidemiology of the disease, it cannot be left out of account. I have attempted to avoid the difficulty by restricting the comparison to the contacts of cases notified in March and later months only. This limitation reduces the numbers to seventy inoculated persons with no cases, and thirteen uninoculated persons with two cases; a distribution which, if less improbable than the previous one, still has the respectable odds of nearly ninety-nine to one against it (measured by Pearson's test). I think, therefore, that the objection does not deprive the comparison of value as evidence even if it remains in general terms unanswered.

The second criticism is that we have no details of the *length* of exposure to risk in the individual cases forming our data. Upon any hypothesis, length of exposure, whether to the possibly infective patient or to the environmental conditions which favour the disease, must be of the essence of the matter. Had we been dealing with an absolutely large experience we might perhaps have assumed that individual variations of exposure would average out and that one sample would not be more favourably circumstanced than the other. We have indeed no evidence that there *was* any such distinction, but since the total numbers are small, and all that we have arithmetically proved is that random variations are unlikely to have given rise to the observed difference, we can hardly neglect the possibility of material variations—other than the fact of being or not being inoculated—having played a part.

If the results obtained are weighed against the objections cited, I think the following conclusion may be drawn. The experience of the vaccinated contacts was more favourable than that of the unvaccinated contacts. The difference is too great to be dismissed as a mere error of sampling and cannot be explained by differences of age constitution or closeness of association. Variations of infectivity and length of exposure cannot, however, be eliminated and *may* account for the result, but there is no evidence that they do so.

One further trial remains to be described. In a certain number of families a proportion of the members were inoculated before the disease appeared among them. In all fifteen families returned cases after some 10 per cent. of the inhabitants of Salisbury had been inoculated (Table V). If we regard these families as fifteen bags each containing certain numbers of black (inoculated) and white (uninoculated) balls, and draw at random one ball from each what is the chance that all the balls drawn will be white? I find that the chance is 0·163 or odds of a little more than five to one against. This result is also, so far as it goes, in favour of inoculation.

TABLE V.—FAMILIES IN WHICH CEREBROSPINAL FEVER OCCURRED AFTER 10 PER CENT. OF THE SALISBURY POPULATION HAD BEEN INOCULATED, WITH PARTICULARS AS TO THE NUMBERS IN EACH FAMILY INOCULATED BEFORE THE DISEASE BROKE OUT.

Reference	Number uninoculated	Number inoculated
Pe.	3	—
Ba.	6	1
Pa.	7	2
Cr.	7	—
Sk.	2	2
Sy.	7	—
Lo.	5	—
Ha.	10	—
Sw.	6	1
Ro.	4	3
As.	6	—
Wi.	6	—
Rb.	3	
Cl.	4	—
Ho.	7	—

Lastly, a circumstance deserves mention which, although not within the purview of a statistical analysis, is of some significance. During the epidemic season of 1915-16, cerebrospinal fever did not become epidemic at Salisbury. It does not follow that because in one year there was an epidemic we should expect a recurrence in the following year; indeed, in the case of some diseases exhibiting a periodicity with an amplitude greater than twelve months, such an event would be unlikely. But in the case of cerebrospinal fever the experience of other towns suggests that when once introduced it rarely dies out within a year, so that it is open to anyone to attribute the recent immunity of Salisbury to the effects of last year's vigorous inoculation campaign. The only comment I have to make on this argument is that it seems to me quite as convincing as most of the arguments to be found in bacteriological treatises upon cerebrospinal fever.

The results of this inquiry will now be summarized. In the first place, it appears that the form of the epidemic wave on the civil population differed significantly from that characterizing the outbreak among troops occupying the surrounding districts. This difference corresponds to the establishment of a sensible proportion of inoculated persons among the population and may be causally connected therewith. The statistics can, however, only establish a correspondence, not a causal connexion, and the value of the correspondence is weakened by the facts that we are working with very small numbers, that our knowledge of the epidemic form of the disease is incomplete and that we cannot even exhaustively enumerate the factors which may have co-operated in producing the result.

In the second place, the attack-rates upon the inoculated and uninoculated populations as wholes and at certain age groups are favourable to the former, but, even if significance attaches to such comparisons in general, the absolute magnitudes here involved do not lead to results beyond the limits of probable chance fluctuations.

In the third place, the after-histories of contacts yield data suggesting a utility in inoculation. This evidence is open to certain objections but is of real value.

In the fourth place, the distribution of the disease between inoculated and uninoculated members of families reporting cases after the widespread introduction of inoculation is favourable to the inoculated, but the numerical measure of the advantage is not decisively great.

The combined effect of these results is not overwhelming but is sufficient to justify the following conclusion—viz., that a *prima facie* case in favour of prophylactic inoculation has been made out. It appears that general inoculation may be an efficient weapon in our struggle against cerebrospinal fever as an epidemic disease.

From the point of view of the epidemiologist, it is desirable that an adequately controlled experiment on a large scale should be undertaken. Failing this, a complete analysis of the military data should be carried out with the object of providing controls on future results derived from inoculated populations. In particular the time-curve of an epidemic, the case-rate amongst close contacts, especially under conditions approximating to those of civil life, and the curve of age susceptibility require to be investigated in greater detail and on the basis of far larger numbers than has so far been possible.

The notion that epidemiology is an occupation for the leisure moments of a bacteriologist is especially prevalent in connexion with cerebrospinal fever. Dr. Hamer's paper may help to dispel this illusion, which is my excuse for the publication of these notes. I wish to add the expression of my sincere thanks to Dr. Fison, Medical Officer of Health of Salisbury, Mr. G. F. Fowles, Sanitary Inspector, and the various medical practitioners in Salisbury, who have provided data for the statistical analysis.

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[3] Greenwood and Yule. *Proc. Roy. Soc. Med.*, 1915 (Epid. Sect.), p. 113.

[4] Pearson. *Biometrika*, 1911, viii, p. 250.

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ON THE STATISTICAL INTERPRETATION OF SOME BACTERIOLOGICAL METHODS EMPLOYED IN WATER ANALYSIS.

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(With 3 Charts.)

We were recently consulted by an officer serving on the Western Front as to the significance attaching to ordinary bacteriological methods of gauging the potability of waters. He wished to know what was the probability that a given water supply did not contain more than a certain proportion of bacteria in the unit volume, it having been found that particular samples tested showed no growth while, perhaps, larger samples had done so, or that so many out of a series of samples of the same size had given positive results. Having obtained what seemed to us a reasonable solution of the particular problem proposed, we thought that the results might interest other officers and bacteriologists who have to do similar work. A survey of the criteria actually used by bacteriologists when they form an opinion as to the purity of waters seems to us to emphasise the need for some discussion.

So far as we can learn, the standard method is to find the minimum quantity of water from which a culture of the organism in question is obtained, usually at a single trial. It is also generally held that a useful indicator of pollution is furnished by the *B. coli* group; the difference between such highly refined techniques as that of the Metropolitan Water Board's experts and the rapid Field Service method introduced by Lieut.-Colonel P. S. Lelean is that the former envisage a carefully defined and limited group of organisms, while the latter merely determines the presence of such as ferment lactose, when grown in MacConkey's bile salt broth, within 24 hours. It will be remembered that Houston has expressed positive results in terms of the numbers of tubes containing "flaginacs," *i.e.* organisms which:

(1) give greenish fluorescence (fl) in neutral-red broth,
(2) acid and gas (ag) in lactose-peptone,
(3) indol (in) in broth,
(4) acidity (ac) and clotting of litmus-milk.

Whether this rigorous examination really excludes many organisms which, although passing the lactose fermentation test, ought not to be admitted into the *B. coli* fold, is a question we are incompetent to answer.

With respect to standards, Dr Savage, who has worked in the Metropolitan Water Board's laboratory, writes that any deep well or spring water which contains *B. coli* (rigidly defined by such methods as that above detailed) in a sample of 100 c.c. or less should be regarded with great suspicion. In the case of surface supplies and shallow wells, he writes: "If no *B. coli* are present in 50 c.c., the water may probably be safely passed as satisfactory, as far as conditions actually present are concerned." "For rivers used as sources of drinking water, without artificial purification, similar standards are applicable[1]."

Colonel Lelean's standard is not defined. In practice he appears to have used 7 tubes containing respectively 20, 15, 10, 5, 2, 1 and ½ c.c. of water to be tested[2]. The total volume of water so used is said to have been 50 c.c. (in reality, as it appears, 53½ c.c.) and the tests "giving all negatives were recorded as having fractors in 75 c.c. instead of 50 c.c., while the all-positive results were given a value of fractors in ¼ c.c. instead of ½ c.c." This novel statistical approximation does not, however, seem to have been uniformly employed by Colonel Lelean, for the number 50, and also the number 25, is frequently to be found in the column of his tables headed "Minimal number of c.c. containing lactose fractors." As no single tube of his series contained either 50 or 25 c.c. it is not obvious how these results were reached.

Nor is Colonel Lelean the only writer from the perusal of whose lucubrations the student may rise with some sense of bewilderment. Professor Hewlett[3] states that for the examination of an ordinary drinking water he usually employs five tubes with 1 c.c. of the water in each, two tubes (double strength) with 10 c.c. in each, and one tube (double strength) with 25 c.c." (p. 584). Then later he remarks (p. 587) "The detection and enumeration of *B. coli* are regarded by all as perhaps the most important part of water examination. The number of *B. coli* is estimated from the amounts of water that have been added to the tubes of media, which, however, assumes that the organism is regularly distributed throughout the sample, and this must so far as possible be ensured by thorough mixing. The results generally come out fairly concordantly, though irregularities exceptionally occur which can only be obviated by making duplicate sets of cultures. It is better to state the result as "*B. coli* present in ... c.c. of water" rather than to say that so many *B. coli* are present, though as a matter of fact the latter statement is approximately correct. Adopting the writer's method for *B. coli* (p. 584), if *none* of the tubes contains *B. coli*, we say that "*B. coli* is absent from 50 c.c."; if the 25 c.c. tube contains *B. coli*, but not the remainder, "*B. coli* is present in 25 c.c. but not in less, and so on."

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[1] Savage, *The Bacteriological Examination of Water Supplies*, pp. 185–6. London, 1906.

[2] Bacteriological Examination of Waters in the Field. *Journ. Royal Army Medical Corps.* Sept. 1914.

[3] *Manual of Bacteriology*, 5th Edition. London, 1914.

"If nothing is known about the water, the following standards may be adopted:

(*a*) *Waters of good quality.* *B. coli* absent in 50 c.c. of the water.

(*b*) *Waters of medium quality.* *B. coli* present in 50 c.c. but absent in 25 c.c.

(*c*) *Waters of poor quality.* *B. coli* present in 50 c.c. and 25 c.c., but absent in 10 c.c.

(*d*) *Waters of suspicious quality.* *B. coli* present in 50 c.c., 25 c.c. and 10 c.c., but absent in 1 c.c.

(*e*) *Waters unfit for drinking.* *B. coli* present in 1 c.c. or less."

In following paragraphs there is some qualification of the standards here laid down, it being pointed out that in upland surface waters a high degree of contamination may only be due to pollution by the excreta of animals and therefore not dangerous, while in the case of spring or deep well water *B. coli* should be absent from at least 50 c.c.

In the passage cited from p. 587 it is not explained how "the number of *B. coli* is estimated from the amounts of water that have been added to the tubes of media" even on the assumption of regular distribution, nor how any amount of stirring can ensure regular results, any more than stirring up the tickets in a bag containing equal numbers of black and white cards can ensure that one will always draw five black and five white out of ten. It is not clear how duplicate sets of cultures can obviate irregularities, when two or more sets may give different results. The student will agree that when he fails to get a positive in any one of the author's series of tubes "*B. coli* is absent from 50 c.c." But if the 25 c.c. tube gives a positive but none of the remainder, he may legitimately object to the statement "*B. coli* is present in 25 c.c. but not in less," seeing that what has really been found is that *B. coli* is present in one lot of 25 c.c. but not in another—the lot made up of five 1 c.c. tubes and two of 10 c.c. The concluding words of the paragraph "and so on" also open up a vista of possible questions. If one of the 10 c.c. tubes gives a positive and the other a negative, how is the result to be stated? If one, or two, or more of the 1 c.c. tubes give positives, but not the remainder, what conclusion is to be drawn? Passing then to the standards laid down under heads (*a*)—(*e*), how is the student to determine standard (*b*) from the author's series of tubes? If *B. coli* is "present in 50 c.c." in his series, it is most likely to be present in the 25 c.c. tube and is therefore not absent from 25 c.c.: if present in the 25 c.c. tube, it is also very probably present in one of the others which total to 25 c.c. and this makes the case worse. Standard (*b*) cannot therefore be determined from the given series of tubes. Standard (*c*) also fails: if none of the 10 c.c. or 1 c.c. tubes give a positive, the actual result is, as already pointed out, "*B. coli* present in one sample of 25 c.c. but not in another." If either of the 10 c.c. tubes give a positive, one cannot state that *B. coli* is absent from 10 c.c. Precisely similar criticisms apply to (*d*). If *B. coli* is absent from all the 1 c.c. tubes, it is absent from 5 c.c., not from 1 c.c. If any one of the 1 c.c. tubes give a positive, it is not "absent from 1 c.c."

The train of reasoning followed in the treatise of Colonel Beveridge and Major Wanhill is somewhat different. These authors employed the usual MacConkey medium and they classified waters in practically the same way as Savage, *e.g.* they held that the absence of *B. coli* from 100 c.c. indicated a very pure water, while presence in 100 but absence from 50 c.c. "is an indication of a good water, which has been polluted in some way, by animal or by human excreta, but in such small amount, or at such a distant period that there would not be much danger attaching to the use of such water for a town supply, if filtered" (p. 152). They further observed that "For temporary camps, if inspection could reveal the presence of no polluting agency and it could be surmised that the excreta of sheep or cattle were the cause, the presence of *B. coli* in

10 c.c. or less might be allowed, this number being often found in moorland streams, where human contamination is unlikely[1]."

Beveridge and Wanhill also provide an arithmetical illustration of the method of determining the density of bacilli in the source from an examination of samples each of the same volume. They say that if ten tubes each inoculated with 1 c.c. of water yielded three positive and seven negative results the sample may be considered to contain *B. coli* in every 3 c.c. of water, adding: "this is a rough estimate and is not mathematically accurate." We may observe that it was this somewhat delphic utterance which induced an officer on water duty to submit to us the problem which was the starting point of the present investigation.

Before detailing our investigation, we may emphasise certain considerations which were, no doubt, present in the minds of the various authors cited but seemed to them too trivial to state.

The fact that a given volume of water tested contains no bacilli, or none which will grow, does not prove that the source of supply is sterile, the point is merely that the greater the volume tested with negative results the smaller is likely to be the population of organisms existing in the supply; none of the writers has attempted to provide a scale of bacterial densities corresponding to the increase of the minimum quantity of water found sterile on examination. We think, indeed, that the tenor of the passages cited creates a presumption that the authors' criterion really is that sources *shown by other methods or found from practical experience* to be safe or to be unsafe have *usually* been found to give sterile readings when samples of the assigned size have been tested. This would explain, for instance, the lower standard adopted in the case of moorland waters. This is undoubtedly a reasonable attitude of mind enough, but it is necessary to remark that the process is not wholly satisfactory, since two observers both testing the same source on, say, the basis of a sample of 100 c.c. might obtain the one a positive, the other a negative result, so that the one would reject and the other pass the supply. Further, no criterion is provided of the increase in accuracy of prediction attained when two, three or more samples of 100 c.c. all give sterile readings.

The object of this paper is to provide such criteria or at least to indicate the method by which they may be obtained in any given case. The actual technique employed is so different in detail in different cases (thus the Metropolitan Water Board use tubes with volumes in decreasing geometrical progression, 100 c.c., 10 c.c., 1 c.c., etc., while Colonel Lelean had tubes of diminishing volume but not diminishing uniformly), that it is not possible to provide a table of standards which will be of use to all observers. Such tables can be drawn up, with the help of general formulae obtained, but, as the arithmetic if simple is laborious, we have confined ourselves to the provision of a few illustrative examples.

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[1] Beveridge and Wanhill, *The Sanitary Officer's Handbook of Practical Hygiene*, 2nd Edition. London, 1912.

SECTION I. PRELIMINARY PROPOSITIONS.

If in the water from which samples of, say, 1 c.c. each are drawn there exist B bacilli in all in a total volume of W c.c. of water, then, the distribution of bacilli being assumed to be random, the probable numbers of c.c. with 0, 1, 2, 3,... bacilli in each are given by the binomial expansion of

$$\left(\frac{W-1}{W}+\frac{1}{W}\right)^{B} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(1).$$

Since B and W are both very large indeed, (1) becomes by a well-known transformation originally given by Poisson[1]:

$$e^{-\lambda}\left(1+\lambda+\frac{\lambda^2}{2!}+\frac{\lambda^3}{3!}\ldots\ldots\right) \quad \ldots\ldots\ldots\ldots\ldots\ldots(2)$$

where $\lambda=\frac{B}{W}$. The problem then reduces itself to that of determining the appropriate value of λ and the probable reliability of its determination.

SECTION II. CASE OF A SINGLE TEST.

We first consider the case of a sample of N c.c. having been taken and found sterile.

The chance of this happening for a given value of λ, is, by (2), $e^{-N\lambda}$.

Now it is reasonable to assume that all values of λ from 0 to some upper limit w are *a priori* equally probable (by analogy with Bayes' postulate) so that the chance of λ being within the range $\lambda \pm \frac{1}{2}d\lambda$ is $d\lambda/w$ and the chance that, λ being within the range, the event happens is $e^{-N\lambda}$, so that the complete chance of λ not exceeding some assigned value κ is:

$$P=\frac{\int_0^{\kappa} e^{-N\lambda}\,.\,d\lambda}{\int_0^{w} e^{-N\lambda}\,.\,d\lambda}=\frac{1-e^{-N\kappa}}{1-e^{-Nw}}.$$

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[1] *Recherches sur la Probabilité d. Jugements, etc.*, p. 190 and p. 206.

e^{-Nw} diminishes very rapidly as w increases and we may put $w = \infty$ so that the required chance is

$$P = 1 - e^{-N\kappa} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3).$$

Consequently if we desire to assign an upper limit to the probable density of bacilli in a source of supply a sample of N c.c. from which has proved sterile we have the following simple method. Suppose we take as our standard the limit corresponding to odds of 99 to 1.

Then, from (3)

$$e^{-N\kappa} = \cdot 01,$$

and the odds are 99 to 1 that κ is not greater than

$$-\frac{\log \cdot 01}{N \log e}.$$

Looking at the matter from the standpoint of the frequency distribution, we may say that the frequency distribution of bacilli per c.c. in waters which give a negative result on testing N c.c. is

$$y = N \,.\, e^{-N\lambda}.$$

The maximum is at zero and the curve tails off rapidly towards the higher densities. The actual curve for $N = 100$ is shewn in Fig. 1.

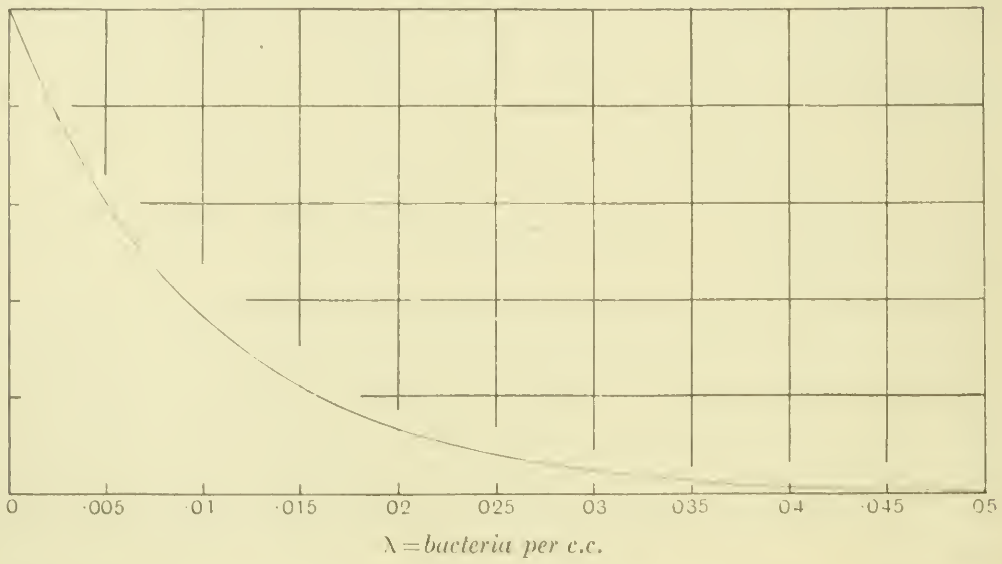


Fig. 1. Frequency distribution of bacterial densities when 100 c.c. give a negative result. The area of the curve is 10 squares.

Section III. Case of Two Tests.

We now take the instance of two tubes having been used with respectively n and m c.c. of water in each. Should both prove negative, we reach the previous result putting $n + m = N$. If the n c.c. tube is negative and the m c.c. tube positive then from (2) by similar reasoning we have for the chance that λ does not exceed κ:

$$P = \frac{\int_0^\kappa e^{-\lambda n} (1 - e^{-\lambda m})\, d\lambda}{\int_0^w e^{-\lambda n} (1 - e^{-\lambda m})\, d\lambda},$$

putting w = infinity, as before, we have

$$P = 1 - \left(\frac{n + m}{m} e^{-n\kappa} - \frac{n}{m} e^{-(n+m)\kappa} \right) \quad \ldots\ldots\ldots\ldots\ldots(4).$$

Taking again some particular value of odds, such as 99 to 1, (4) enables us to find a value of κ.

It is to be noted that the method breaks down if *both* samples give positive results for

$$\frac{\int_0^\kappa (1 - e^{-n\lambda})(1 - e^{-m\lambda})\, d\lambda}{\int_0^w (1 - e^{-n\lambda})(1 - e^{-m\lambda})\, d\lambda},$$

vanishes for $w = \infty$, so that no upper limit is assignable to λ.

The problem of this section may also be studied from the point of view of curve fitting. We have, writing $m + n = N$,

$$y = \frac{Nn}{m} e^{-\lambda n} (1 - e^{-\lambda m}) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(5),$$

for the distribution of λ, since the total area is

$$\int_0^\infty (1 - e^{-\lambda n})(1 - e^{-\lambda m})\, d\lambda = \frac{m}{Nn}.$$

The curve extends from 0 to ∞, and differentiating (5) with respect to λ and equating to zero we find for the mode

$$\lambda = \log \frac{N}{n} \cdot \frac{1}{m \log e} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(6).$$

Now

$$\int_0^\infty x^n e^{-x\kappa} \,.\, dx = \frac{n}{\kappa} \int_0^\infty x^{n-1} \,.\, e^{-x\kappa} \,.\, dx = \ldots = \frac{n\,!}{\kappa^{n+1}}$$

(integrating successively by parts and noticing that the first part vanishes), so that the successive moments of (5) are determinate. We have:

$$\text{mean} = \frac{N+n}{nN} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(7),$$

$$\mu_2 = \frac{N^2+n^2}{n^2 \, . \, N^2} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(8),$$

$$\mu_3 = \frac{2\,(N^3+n^3)}{N^3 n^3} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(9).$$

This curve therefore has positive skewness for all finite values of n, *i.e.* the long tail of the distribution extends to high values of λ.

Again if we write λ_1 for the modal value found in (6), the chance that the true bacterial density does not exceed the modal value is:

$$1 - \frac{Nn}{m} \int_{\lambda_1}^{\infty} (e^{-\lambda n} - e^{-\lambda N})\, d\lambda \quad \ldots\ldots\ldots\ldots\ldots\ldots(9\,a),$$

integrating and putting λ_1 equal to (6), (9 *a*) becomes:

$$1 - \frac{1}{mN} \left(\frac{n}{N}\right)^{\frac{n}{m}} (N^2 - n^2) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots(10).$$

If $m = \rho n$, $N = (\rho + 1)\, n$, (10) becomes:

$$1 - \frac{1}{\rho\,(\rho+1)\,n^2} \left\{\frac{1}{(\rho+1)}\right\}^{\frac{1}{\rho}} \{(\rho+1)^2 n^2 - n^2\} = 1 - \frac{\rho+2}{(\rho+1)^{\frac{\rho+1}{\rho}}} \ldots(11).$$

If ρ becomes very large the fraction approximates to unity and most of the area lies beyond the mode. This is reasonable, for the information that a second sample of infinite size gave a positive result adds nothing to our knowledge and (4) reduces to the case of the last section, for which, as λ cannot be negative, the mode is at $\lambda = 0$.

As an illustration of the forms that frequency distributions may take, the curves have been calculated (1) for the case in which 100 c.c. gives a positive result and 50 c.c. a negative, (2) for the reciprocal case in which 100 c.c. gives a negative but 50 c.c. a positive—an inconsistent but perfectly possible result. The two curves are shewn together in Fig. 2. Both distributions are very skew. The respective modes are $\lambda_1 = 0{\cdot}011$ and $\lambda_1 = 0{\cdot}0081$.

The question naturally arises, what is the probability of an inconsistence such as that assumed in the second illustration? If n c.c. have given a negative result what is the probability that a subsequent sample of m c.c. from the same water will also give a negative, or on the other hand a positive? If n c.c. have given a negative, the probability that λ lies within the limits $\kappa \pm \frac{1}{2} d\kappa$ and that a second sample of m c.c. will then give a negative is $n e^{-N\kappa} d\kappa$, where $N = m + n$ as above. For all values of λ the required probability is therefore the integral of this from 0 to ∞ or:

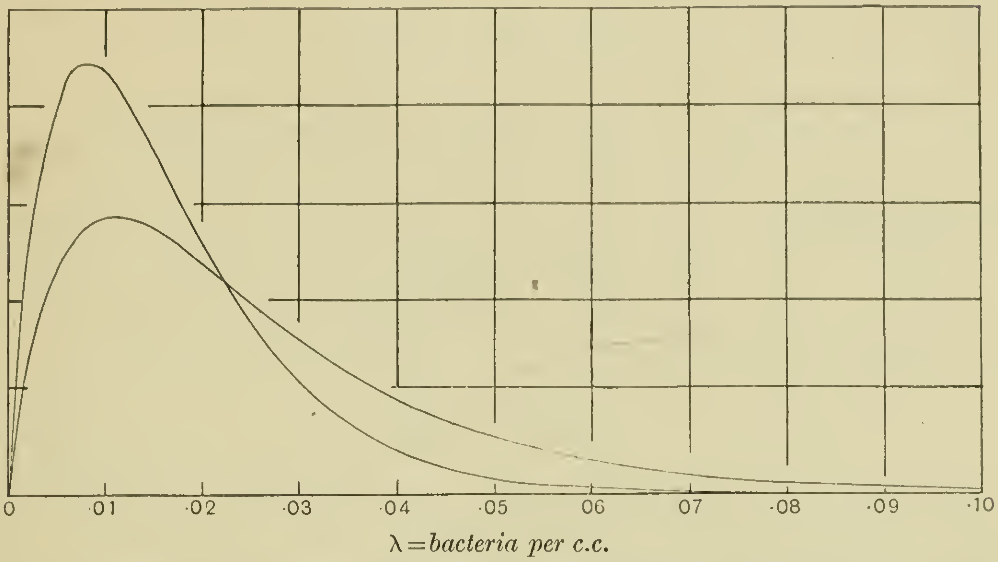


Fig. 2. Frequency distributions of bacterial densities for (1) 100 c.c. +, 50 c.c. −, the curve with the lower maximum, (2) 100 c.c. −, 50 c.c. +, the curve with the higher maximum. The area of each curve is 10 squares.

$$\left.\begin{array}{r}\text{chance that } n \text{ c.c. having given a negative, a second}\\ \text{sample of } m \text{ c.c. will also give a negative}\end{array}\right\} = \frac{n}{N}.$$

If $n = 100$, $m = 50$, the chance is $\frac{2}{3}$ or the chance of an inconsistence no less than $\frac{1}{3}$. If $m = n$, the chance is $\frac{1}{2}$. The results emphasise our previous remarks on the necessity of remembering that all results are subject to considerable fluctuations of sampling.

SECTION IV. CASE OF SEVERAL TESTS.

We first examine the case of N tubes, each containing the *same* quantity of water, which is taken as unity, n of the tubes being sterile while m give positive results.

If follows directly from (2) that the most probable value of λ is given by

$$\frac{N-m}{N} = e^{-\lambda} \text{ or } \lambda = \frac{1}{\log e} \log \frac{N}{N-m} = 2{\cdot}302585 \log \frac{N}{N-m} \quad \ldots (12).$$

The value so obtained is, however, subject to wide fluctuations of sampling as we shall now show.

Calling $\frac{N-m}{N}$, x, we have

$$\sigma_x^2 = \frac{1}{N} \cdot \frac{N-m}{N} \cdot \frac{m}{N}.$$

If $z = \log \frac{1}{x}$, $dz = -\frac{1}{x}\,dx$, $(dz)^2 = \frac{1}{x^2}(dx)^2$,

and consequently

$$\sigma_z^2 = \frac{1}{x^2}\,\sigma_x^2 = \frac{m}{N(N-m)}.$$

And finally the "probable error" of λ determined from (12) is:

$$1{\cdot}553068\sqrt{\frac{m}{Nn}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(13).$$

As will be seen from the probable errors calculated as illustrations in the columns headed 4 and 10 of Table I the fluctuations are such that it seems better to resort to the method adopted in the previous sections.

TABLE I.

Probable number of bacilli per 1000 *c.c. for a given number of blanks out of a given number of test tubes* (1 *c.c. in test*). *If* 10 *c.c. are placed in each tube instead of* 1, *divide all figures by* 10.

No. of blanks	Out of								
	2	3	4	5	6	7	8	9	10
1	693	1098	1386 ± 1345	1609	1792	1946	2079	2197	2303 ± 1473
2	—	405	693 ± 777	916	1098	1253	1386	1504	1609 ± 982
3	—	—	287 ± 448	510	693	847	981	1098	1204 ± 750
4	—	—	—	223	405	560	693	811	916 ± 602
5	—	—	—	—	182	336	470	588	693 ± 491
6	—	—	—	—	—	154	287	405	510 ± 401
7		—	—	—	—	—	134	251	357 ± 322
8	—	—	—	—	—	—	—	118	223 ± 246
9	—	—	—	—	—	—	—	—	105 ± 164

We have accordingly for the chance (P) that λ does not exceed some value κ

$$P = \frac{\int_0^\kappa e^{-\lambda n} (1 - e^{-\lambda})^m \, d\lambda}{\int_0^w e^{-\lambda n} (1 - e^{-\lambda})^m \, d\lambda} \quad \text{.....................(14).}$$

Expanding the brackets, the numerator and denominator can be integrated as before and an equation obtained for the value of x corresponding to a given value of P. For small numbers of tubes there is no difficulty, though the laboriousness of the work rapidly increases with the number of tubes. Alternatively, writing

$$e^{-\lambda} = x, \qquad d\lambda = -\frac{dx}{x},$$

substituting and assuming w to be infinite (14) becomes

$$\frac{\int_{e^{-\kappa}}^1 x^{n-1} (1 - x)^m \, dx}{\int_0^1 x^{n-1} (1 - x)^m \, dx} = 1 - \frac{\int_0^{e^{-\kappa}} x^{n-1} (1 - x)^m \, . \, dx}{\dfrac{(n-1)!\, m!}{(m+n)!}} \quad \text{......(15).}$$

If now we require the value of κ which corresponds to some assigned probability, we can rewrite (15) as

$$\frac{\int_0^x x^{n-1} (1 - x)^m \, . \, dx}{\dfrac{(n-1)!\, m!}{(m+n)!}} = 1 - P \quad \text{..................(16).}$$

Integrating the numerator of (16) by parts, (taking x^{n-1} as the direct integrand so as to keep the terms all positive) it becomes

$$\frac{x^n}{n} \left\{ (1 - x)^m + \frac{m}{n+1} x (1 - x)^{m-1} + \frac{m(m-1)}{(n+1)(n+2)} x^2 (1 - x)^{m-2} \dots \right\} \quad \text{.........(17).}$$

The series converges fairly rapidly and in rough practice it will be found that a sufficiently good approximation is often reached by putting the bracket equal to unity and solving for x from

$$\frac{x^n}{n} = \frac{(n-1)!\, m!}{(m+n)!} (1 - P) \quad \text{.....................(18).}$$

As the sum within the bracket cannot exceed unity, this approximation underestimates x and therefore over-estimates λ, an error on the safe side.

A point of some interest is to consider the problem suggested by (9 *a*) of the last section taking the modal value from (12), *i.e.* putting

$$x = \frac{n}{n+m}$$

(17) then becomes

$$\frac{\left(\frac{n}{n+m}\right)^n}{n}\left\{\left(\frac{m}{m+n}\right)^m + \frac{m}{n+1}\left(\frac{n}{n+m}\right)\left(\frac{m}{m+n}\right)^{m-1} + \frac{m(m-1)}{(n+1)(n+2)}\left(\frac{n}{n+m}\right)^2\left(\frac{m}{m+n}\right)^{m-2} + \dots\right\} \text{......(19)},$$

and in the important special case of $m = n$

$$\frac{(\frac{1}{2})^{2n}}{n}\left\{1 + \frac{n}{n+1} + \frac{n(n-1)}{(n+1)(n+2)} + \frac{n(n-1)(n-2)}{(n+1)(n+2)(n+3)} \dots\right\} \text{.........(19 } a\text{)}.$$

The series in (19 *a*) is readily summed. Call it $F(n)$. Then

$$\frac{2n!}{n!\,n!} \cdot F(n) = \frac{(2n)!}{n!\,n!} + \frac{(2n)!}{(n+1)!\,(n-1)!} + \frac{(2n)!}{(n+2)!\,(n-2)!} + \dots$$

$$= C_n{}^{2n} + C_{n+1}{}^{2n} + C_{n+2}{}^{2n} \dots$$

$$= \frac{1}{2}\overset{2n}{\underset{0}{S}}\, C_r{}^{2n} + \frac{1}{2} C_n{}^{2n}$$

$$= \frac{1}{2} \cdot 2^{2n} + \frac{1}{2}\frac{(2n)!}{n!\,n!}.$$

$$\therefore\ F(n) = \frac{1}{2}\left\{\frac{2^{2n}}{(2n)!} \cdot n! \cdot n! + 1\right\}.$$

$$\therefore\ (19\ a) = \frac{(n-1)!\,n!}{2n!\,2} + \frac{1}{2^{2n+1}n} \text{..............................(19 } b\text{)}.$$

Substituting in (15) we have

$$P = 1 - \left(\frac{1}{2} + \frac{(2n)!}{n!\,n!} \cdot \frac{1}{2^{2n+1}}\right) \text{..................(19 } c\text{)}.$$

Applying Stirling's theorem to the third term it becomes $\frac{1}{2}\frac{1}{\sqrt{\pi n}}$, which is zero when n is infinite, and then the mode divides the frequency into equal parts.

For usual values of n the approximation is slow. Thus

n	Value of (15)	n	Value of (15)
1	·2500	100	·4718
2	·3125	500	·4874
10	·4119		

Reverting for a moment to the integral in the denominator of (16), we may note that its value can be approximated to by Laplace's method of putting

$$\int y \, . \, dx = Y \int e^{-t^2} \frac{dx}{dt} \, . \, dt.$$

Y being the maximum value of the function within the required range, in this case the value for $x = \frac{n-1}{n+m-1}$, if x is written $\frac{n-1}{n+m-1} + \vartheta$, t^2 is obtained as a function of ϑ and then ϑ as a function of t by reversion of series.

The result to a first approximation only is that the integral transforms to

$$\frac{(n-1)^{n-1}(m-2)^m}{(n+m-1)^{n+m-1}} \int e^{-t^2} \frac{\sqrt{2}}{(n+m-1)\left(\frac{1}{m+1} + \frac{m}{(m+2)^2}\right)^{\frac{1}{2}}} \, dt.$$

This method of treatment is, of course, well known. We can, however, employ the method of the last section and investigate the properties of $y = x^{n-1}(1-x)^m$, or the distribution of $e^{-\lambda}$, where the range is from 0 to 1.

The mode is at

$$x = \frac{n-1}{n+m-1} = \frac{n-1}{N-1} \quad \text{.....................} (19\,d)$$

and, the moments about the start of the curve being simply successive B functions we easily find

$$\text{mean} = \frac{n}{N+1} \quad \text{..} (20),$$

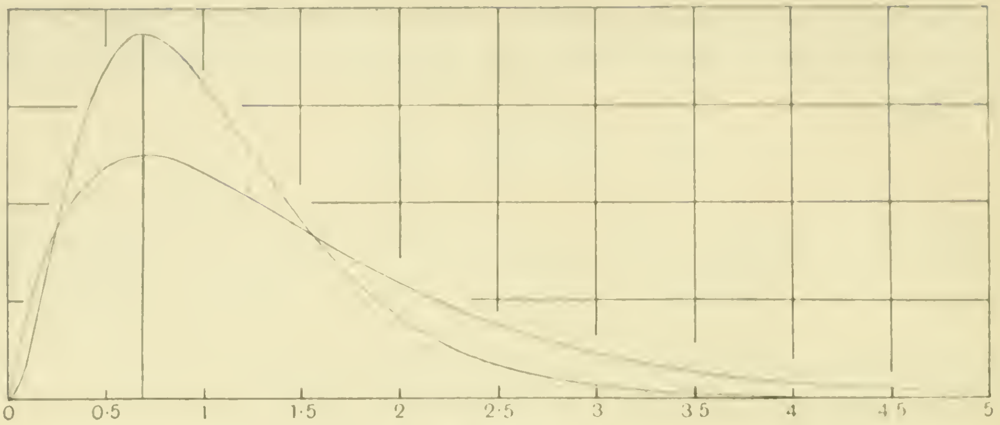
$$\mu_2 = \frac{n(m+1)}{(N+1)^2 (N+2)} \quad \text{..................................} (21),$$

$$\mu_3 = \frac{2n\,[2n^2 - 3n(N+1) + (N+1)^2]}{(N+1)^3 (N+2)(N+3)} \quad \text{.........} (22).$$

A comparison of the mean and mode shews again that the curve is skew for all finite values of n and m greater than zero. The transformed curve, $y = y_0 e^{-\lambda n}(1-e^{-\lambda})^m$ is, of course, also very skew, cf. the two distributions shewn in Fig. 3.

Finally we have the case of samples of different sizes.

Thus if N_1 samples each of a_1 c.c. have given n_1 negative and m_1 positive results, N_2 samples each of a_2 c.c. have given n_2 negative and



λ = *bacteria per c.c.*

Fig. 3. Chart shewing frequency distributions of bacterial densities for 1 positive out of 2 and 2 positives out of 4 (1 c.c. in each test). The area of each curve is 10 squares.

m_2 positive results and ... N_n samples of a_n c.c. have given n_n and m_n negative and positive results, (14) becomes:

$$P = \frac{\int_0^{\kappa} [e^{-\lambda a_1 n_1}(1-e^{-\lambda a_1})^{m_1} \,.\, e^{-\lambda a_2 n_2}(1-e^{-\lambda a_2})^{m_2} \ldots e^{-\lambda a_n n_n}(1-e^{-\lambda a_n})^{m_n}]\,d\lambda}{\int_0^{\infty} [e^{-\lambda a_1 n_1}(1-e^{-\lambda a_1})^{m_1} \,.\, e^{-\lambda a_2 n_2}(1-e^{-\lambda a_2})^{m_2} \ldots e^{-\lambda a_n n_n}(1-e^{-\lambda a_n})^{m_n}]\,d\lambda} \quad \text{.........(23).}$$

This expression does not lend itself to simple treatment in the general case but in any particular case it may furnish a solution without much difficulty. The arithmetic may however prove almost unworkable. In an illustration given in Section V only the most probable values of the bacillary density have been approximately determined by an artifice. If the tests are not repeated, only one test being made with a tube of each size, the expression simplifies.

On the general question of the best series of sizes of tubes to use, when the waters to be tested may present so great a range between purity and the reverse that the use of different sizes seems desirable, we may make a few remarks. One obvious condition, strangely overlooked, is that the size of any one sample should be greater than the sum of the sizes of the smaller samples. Otherwise the observer is simply asking for "inconsistencies" in his results. A geometrical series fulfils the required condition, and the Metropolitan Water Board have actually used a geometrical series with the ratio 10 (0·01, 0·1, 1, 10, 100 c.c.). This ratio seems rather a high one. A geometrical series seems also a natural one to use as the chance of an inconsistence is the same at every point of the series: r being the (ascending) ratio of the series, the chance of an inconsistence between any adjacent pair of samples (the larger giving a negative, the smaller a positive) is $1/(r+1)$.

The various expressions found above enable us to solve all the problems proposed and in the following section we provide a few arithmetical examples of their use.

SECTION V. NUMERICAL ILLUSTRATIONS.

(*a*) *The case of a blank sample.*

If 50 c.c. were tested and found sterile we have from (3)

$$e^{-50\kappa} = 1 - P \quad \text{or} \quad -50\kappa \log e = \log (1 - P).$$

For $P = \cdot 5$ this gives $\kappa = \cdot 01386$.
„ $P = \cdot 99$ „ „ $\kappa = \cdot 09210$.
„ $P = \cdot 999$ „ „ $\kappa = \cdot 13816$.
„ $P = \cdot 9999$ „ „ $\kappa = \cdot 18420$.

So that the chances are even that the source does not contain more than 14 bacilli per litre. It is 99 to 1 that there are not more than 92 per litre, 999 to 1 that there are not more than 138 per litre and 9999 to 1 that there are not more than 184 per litre.

Had 150 c.c., *e.g.* a sample of 100 and a sample of 50 both proved sterile, substitution of 150 for 50 gives for the corresponding values per litre, 5, 31, 46, 62.

(*b*) *One sample is sterile, the other not.*

Suppose 100 c.c. are positive, 50 c.c. sterile. Then using (4) the equation to be solved is

$$1\cdot 5e^{-50\kappa} - \cdot 5e^{-150\kappa} = 1 - P.$$

And we reach:

P	Bacilli per litre	P	Bacilli per litre
·5	21	·999	146
·99	100	·9999	192

(*c*) *Repeated Tests with Tubes of Equal Volumes.*

As a first illustration we take Beveridge and Wanhill's example of 10 samples, each 1 c.c., 7 of which are sterile and 3 show growth.

Here $n = 7$, $m = 3$, $N = 10$ so that by (19 D) and (20) the mode of the x distribution is at ·66667 and the mean at ·63637. That is a source with a density of 405 bacilli per litre (from $e^{-\lambda} = \cdot 66667$) is the most probable state. Owing, however, to the marked skewness of the distribution, this result is of little service, we must solve (16) for different values of $1 - P$.

If we wish to find the density corresponding to $P = \cdot 5$, $\cdot 9$, $\cdot 99$ we have

$$120x^7 - 315x^8 + 280x^9 - 84x^{10} = \left\{ \begin{array}{l} \cdot 5 \\ \cdot 1 \\ \cdot 01. \end{array} \right.$$

Thus, taking the last case, we have

$$f(x) = 120x^7 - 315x^8 + 280x^9 - 84x^{10} - \cdot 01.$$
$$f'(x) = 840x^6 - 2520x^7 + 2520x^8 - 840x^9,$$

and by successive approximation we find that $\cdot 29716$ is very nearly a root.

Hence from $e^{-\lambda} = \cdot 29716$ we reach 1213 bacilli per litre: the odds are 99 to 1 that the density of the source does not exceed this value.

The densities corresponding to an even chance and to odds of 9 to 1 are similarly found to be 439 per litre and 802 per litre respectively.

In Table II we give examples of the significance of one or more sterile tubes in a series of 2–4 samples.

TABLE II.

Tables illustrating the significance of one or more blanks in two, three, or four tests on 1 c.c. If the samples are of n c.c., not 1 c.c., divide the numbers of bacilli per litre by n.

Two tests.

	If the number of blanks is	
The probability is	1	2
0·75 that the bacilli per litre exceed	690	140
0·50 ,, ,, ,, ,,	1230	350
0·25 ,, ,, ,, ,,	2050	690
0·01 ,, ,, ,, ,,	5300	2300
Most probable number per litre	690	nil

Three tests.

	If the number of blanks is		
The probability is	1	2	3
0·75 that the bacilli per litre exceed	990	400	95
0·50 ,, ,, ,, ,,	1580	690	230
0·25 ,, ,, ,, ,,	2390	1120	460
0·01 ,, ,, ,, ,,	5700	2830	1540
Most probable number per litre	1100	410	nil

Four tests.

	If the number of blanks is			
The probability is	1	2	3	4
0·75 that the bacilli per litre exceed	1220	610	210	70
0·50 ,, ,, ,, ,,	1840	950	490	170
0·25 ,, ,, ,, ,,	2670	1410	780	350
0·01 ,, ,, ,, ,,	5990	3170	1960	1150
Most probable number per litre	1390	690	290	nil

As stated at the outset, divergences in the numbers of tubes forming different workers' series are too great to allow us to calculate any single table of general service. But the arithmetical examples should suffice to enable any bacteriologist to construct a table covering his own series.

(*d*) *Tubes of different volumes, test not repeated.*

A series of tubes of different volumes is used by several observers, *e.g.* as already mentioned Lelean, and Hewlett. Comment has already been made on the inconvenient character of the series used in each of these cases. That used by the Metropolitan Water Board is a simple geometrical series with a ratio of 10, viz. 100, 10, 1, 0·1, 0·01 c.c. The ratio is high, but it must be admitted that this simplifies the work of calculating the theoretical significance of the results; we have already pointed out that a geometrical series seems the right series to use.

Writing down equation (23) in its simplified form, where $n_r = 0$ and $m_r = 1$ or conversely, expanding, and retaining only the first power of x in the resulting equation we find the following approximate numbers of bacilli per litre:

	100 +, rest –	100 +, 10 +. rest –	100 +, 10 +, 1 +, rest –	100 +, 10 +, 1 +, 0·1 +
$P = 0·5$	72	720	7260	78940
$P = 0·99$	424	4245	42820	470150
Most probable numbers	23	230	2310	24540

The values of λ are nearly, it will be noticed, but not quite, a geometric series.

(*e*) *Repeated tests with tubes of different volumes.*

This involves the determination of the limiting density from (23). As an illustration we take the following series from one of the Reports of the Metropolitan Water Board.

Size of Sample	Source *A*		Source *B*	
	Negative	Positive	Negative	Positive
100 c.c.	308	30	312	21
10 c.c.	333	5	327	6
1 c.c.	336	2	329	4
·1 c.c.	338	0	333	0
·01 c.c.	338	0	333	0

It seemed difficult to obtain in this case even fair approximations to the values of the bacillary density for given values of P. But the following method gives good approximations to the most probable values. Taking the expression

$$y = y_0 e^{-\lambda(a_1n_1+a_2n_2+\dots)} (1 - e^{-\lambda a_1})^{m_1} (1 - e^{-\lambda a_2})^{m_2} \dots,$$

$$\frac{1}{y}\frac{dy}{d\lambda} = -(a_1n_1 + a_2n_2 + \dots) + \frac{a_1m_1}{1 - e^{-\lambda a_1}} e^{-\lambda a_1} + \frac{a_2m_2}{1 - e^{-\lambda a_2}} e^{-\lambda a_2} + \dots$$
$$= 0$$

for the most probable value. For Series A we find this gives

$$-34503{\cdot}18 + 3000 \frac{e^{-100\lambda}}{1 - e^{-100\lambda}} + 50 \frac{e^{-10\lambda}}{1 - e^{-10\lambda}} + 2 \frac{e^{-\lambda}}{1 - e^{-\lambda}},$$

or writing $e^{-\lambda} = x$

$$2 \frac{x}{1 - x} + 50 \frac{x^{10}}{1 - x^{10}} + 3000 \frac{x^{100}}{1 - x^{100}} = 34503.$$

Clearly x is near unity, as the value on the right is so large. Substitute accordingly $z = 1 - x$ for x and expanding this becomes

$$2 \frac{1 - z}{z} + 50 \frac{1 - 10z + 45z^2}{10z - 45z^2} + 3000 \frac{1 - 100z + 450z^2}{100z - 450z^2} = 34503.$$

Or, ignoring z^2

$$37{,}555z = 37,$$
$$z = 0{\cdot}000985,$$
$$x = 0{\cdot}999015,$$
$$\lambda = 0{\cdot}965 \text{ per litre.}$$

For Series B we find to a similar approximation $\lambda = 0{\cdot}838$ per litre, or the water from Source B is most probably slightly purer than that from Source A, though there is no practical difference.

As a glance at the two series suggests, moreover, they are not consistent with each other. Source B, if the better water, should give fewer positives in the small tubes as well as the large. Actually it gives much fewer in the large tube, but more in the small ones. Using the above values of λ, we find for the theoretical as against the actual distributions of positives:

Size of	Source *A*		Source *B*	
sample	Actual	Calculated	Actual	Calculated
100	30	31·7	21	27·0
10	5	3·3	6	2·8
1	2	·3	4	·2

It looks as if we were not dealing with a mere chance distribution from water essentially of constant character, but with occasional slight contaminations of the source.

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N° 5

XXIV. ON THE CONCENTRATION OF ANTITOXIC SERA.

By ANNIE HOMER.

From the Lister Institute, Elstree.

(*Received May 22nd, 1916.*)

Of the various methods which have from time to time been proposed for the concentration of sera, those developed by Banzhaf have been most widely adopted on a commercial scale. In this communication it is only necessary to refer to:

A. *The Banzhaf-Gibson* [1907] *Two Fraction Method*, in which serum or plasma, diluted with one-third its volume of water, was heated to a temperature of 57° for 15 hours, or of 58° for 7 hours. The liquid was then made 30 per cent. of saturation with ammonium sulphate[1] and the ensuing precipitate (Fraction I) separated by filtration.

The Fraction I precipitate was suspended in saturated brine solution for 48 hours or longer. The liquid was filtered from the insoluble euglobulin and the filtrate, containing in solution the pseudoglobulin and antitoxin which had been present in the Fraction I precipitate, was made 0·25 per cent. with glacial acetic acid. The ensuing precipitate was filtered, pressed and dialysed.

The filtrate from Fraction I was made 54 per cent. of saturation with ammonium sulphate and the precipitate (Fraction II) thus formed was filtered, pressed and dialysed.

The dialysis residues from Fractions I and II were mixed, the requisite amount of salt and preservative added, and finally filtered ready for distribution. This method has been used in these laboratories for some considerable time and it has been found that by this process a good end product can be obtained which remains clear for years. Care must, however, be taken in the first precipitation, as otherwise some of the insoluble globulin passes on and appears in the final product which, in this case, does not remain clear.

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[1] Throughout this paper the phrase "made x per cent of saturation with ammonium sulphate" has been used to indicate the following: To $100 - x$ parts of the serum by volume there were added x parts by volume of a saturated aqueous solution of ammonium sulphate.

B. *Banzhaf's* [1913] *One Fraction Method*, in which serum or plasma, diluted with half its volume of water and made 30 per cent. of saturation with ammonium sulphate, is brought up to a temperature of 60°, the entire heating process taking about two hours.

According to Banzhaf the filtration of the hot serum mixture should proceed rapidly. The precipitate of euglobulin is pressed well or is washed with 33 per cent. saturated ammonium sulphate; the washings are filtered and added to the main bulk of the filtrate which is then made 50 per cent. of saturation with ammonium sulphate. The ensuing precipitate, consisting of pseudoglobulin and antitoxin, is filtered, pressed, dialysed, and the dialysis residues treated in the same way as in the Banzhaf-Gibson process.

Banzhaf considers that, during the shortened heating of the mixture in the new method, a certain amount of pseudoglobulin is converted into euglobulin, and bases this conclusion on his own experiments [1908]. The effect of heating the serum mixtures is thus to ensure the precipitation of an amount of euglobulin which ordinarily would require a higher percentage of ammonium sulphate for precipitation, and which does not, as would happen in the latter case, carry down with it an appreciable amount of pseudoglobulin and antitoxin.

Banzhaf claims that the new process possesses the following advantages over the former Banzhaf-Gibson process.

(1) There is only one finished product for dialysis.

(2) There is a considerable saving of time for the reason that the first precipitate (30 per cent. of saturation with ammonium sulphate), instead of being subjected to the more lengthy process of extraction with saturated brine and subsequent precipitation of the brine extract with acetic acid, is washed with 33 per cent. ammonium sulphate and the washings added to the main filtrate.

(3) The expenses incurred in the process are considerably less, both as regards labour and the use of precipitating materials, etc.

This new method certainly possesses the advantage of being a much shorter process than the former Banzhaf-Gibson method, but unfortunately it is not always easy to work. The difficulty that sometimes arises in carrying out the process is that the serum will not filter well. This difficulty has arisen in the course of my work both in an English and in a Canadian laboratory; in the latter laboratory I had the advantage of consulting Dr Banzhaf personally about the matter. He suggested that a slight alteration in temperature or in the concentration of ammonium sulphate might be tried to overcome the difficulty, but these suggestions, although of value in some cases, did not always provide a satisfactory solution to the problem. The majority of workers on the subject with whom I have discussed the question have been faced with the same irregularity in concentrations by Banzhaf's new method.

Practical experience shows that the production of a readily filterable end product which will remain almost permanently clear, goes hand in hand with the rapid filtration of the serum mixtures after the preliminary heating process. When the heated mixtures filter with difficulty it is in some cases necessary to allow the dialysed pseudoglobulin-antitoxin precipitate to stand in the ice chest for weeks in order that the opalescent suspension therein may settle—otherwise filtration through filter candles is accomplished with great difficulty and, moreover, the final product will not remain clear.

This delay is a great disadvantage, and is one which nullifies any gain in time which should accrue from the fact that there is only one fraction to be considered.

It was obvious that the technique of the new process called for improvement or for enlargement, and in this laboratory we have ascertained that the above mentioned irregularities in the filtration can be obviated by the addition of sodium chloride to the serum mixtures, as described below.

The serum or plasma is diluted with one-half its volume of water, made 30 or 31 per cent. of saturation with ammonium sulphate and to the mixture is added 1·5 per cent. of solid sodium chloride. The temperature of the mixture is gradually raised to 61°, 63° or even 65° and kept at this point for a few minutes only. The mixture is allowed to cool to 40°–45° and filtered.

In every case the filtration of this hot mixture and of the subsequent 50 per cent. saturation proceeds rapidly. The liquid obtained after dialysis of the pseudoglobulin-antitoxin precipitate shows no sign of the presence of an opalescent colloidal suspension. After filtration through muslin it is as clear to the naked eye as though it had already been passed through paper pulp. On standing in the ice box these dialysis residues remain clear as there is nothing in opalescent suspension to be deposited.

On the addition of cresylic acid or tricresol to the dialysis residues a certain amount of precipitate is formed in all cases. In the batches of concentrated serum to which the 1·5 per cent. of solid sodium chloride has been added this deposit is exceedingly slight, even after the serum has stood many weeks. Such a serum may undergo its final filtration through pulp and filter candles almost at once: the end product will remain perfectly clear.

By the addition of 1·5 per cent. solid sodium chloride to the serum mixtures we have so far obtained a satisfactory end product with certainty and the loss of antitoxic units has been very small; a most important item to be considered in assessing the value of any method. From incomplete experiments we have reason to believe that by a further study of the dilution of the plasma, of the concentration of the ammonium sulphate and of sodium chloride and of the general treatment of the mixture, we may be able to reduce the loss of antitoxin to a minimum and at the same time produce a much better end product.

At Dr MacConkey's suggestion, an effort has been made to control the heating of the serum by means of the immersion refractometer, in order to see if some easy indication might be obtained as to the best point at which to interrupt the process. This appeared hopeful in view of Banzhaf's explanation of the effect of heating. However, the results obtained from the use of this instrument, of which the reliability and accuracy have been well established, have demonstrated that no useful indication was forthcoming, for, contrary to Banzhaf's hypothesis, during the heating process there is no appreciable change in the amount of protein in solution in the serum mixture. This result, not entirely unsuspected, has led to some interesting investigations which have been carried out with routine and with experimental batches of serum in order to elucidate:

I. The value of the heating process in the new One Fraction Method.

II. The part played by the sodium chloride in improving the technique of Banzhaf's new process.

I. *The value of the heating process in the Banzhaf new One Fraction Method.*

As previously stated, it was originally hoped that a study of the refractive indices of the filtrates of samples taken from the serum mixtures from time to time during the heating process would indicate the point at which optimum conditions for filtration had been reached. With this object in view the following procedure was adopted.

Routine concentrations were carried out in which serum or plasma, diluted with one-half its volume of water, and made 30 per cent. of saturation with ammonium sulphate, was heated to a temperature of 61°, 63°, 65° and kept at the specified temperature for a few minutes only, the process taking from 2 to 3 hours in all. Samples of the original plasma and of the plasma mixtures at various stages during the heating process were taken. The samples were filtered and the refractive indices of the clear filtrates were measured by means of the Zeiss Immersion Refractometer. In each case the clear liquid, thus examined, was boiled and filtered and the refractive index of the second filtrate which contained the serum salts and ammonium sulphate in solution, was noted. The amount of protein in solution in the serum mixture was calculated from the difference between the values of the refractive index of the whole serum mixture and of the filtrate containing the serum salts.

TABLE I.

Oxalated plasma diluted with half its volume of water and made 31 *per cent. of saturation with ammonium sulphate. The mixture was heated to* 63° *and kept at that temperature for about five minutes.*

Protein Content of the Original Plasma = 7·45 per cent.

		Scale Divisions of Refractometer Measurements of:		
Duration of time of heating of plasma mixtures	Temperature of mixtures	(*a*) The filtrates from the mixtures	(*b*) The filtrates (*a*) diluted with an equal volume of water, boiled and filtered	Per cent. of protein remaining in solution expressed per 100 cc. of the original plasma
0	16°	98·65	51·50	5·11
1 hr 50′	53·5°	98·72	51·50	5·15
2 hrs 5′	57°	98·55	51·40	5·16
2 hrs 20′	59°	98·56	51·50	5·16
2 hrs 40′	61°	98·50	51·60	5·09
2 hrs 45′	62°	98·85	51·70	5·11
2 hrs 55′	63°	98·90	51·60	5·13

These results have been calculated from the values given in Reiss' table [1904]. For purposes of comparison it has been assumed throughout this research that in serum mixtures containing from 0 to 4 per cent. of protein the difference in protein content for 1 scale division = 0·220 per cent.; from 4 to 8 per cent. the difference for 1 scale division = 0·2175 per cent.

Brailsford Robertson [1915] considers that Reiss' figures are somewhat high, but since in the present investigation the *relative change* only in the protein content of the serum mixture is to be investigated it follows that it is quite immaterial whether Reiss' values are above or below the true value.

An examination of the results from a typical concentration given in Table I shows that during the heating process there was no appreciable change in the refractive indices of filtrates of the serum mixtures heated from room temperature to 63° or 65° and kept at these latter temperatures for

5 or 10 minutes. The very slight changes observed may have been due to the effect of heating the serum itself (*post* p. 301 and Table XIV).

Banzhaf seems to consider that the value of the heating lies in the conversion of soluble pseudoglobulin into a further amount of euglobulin, and that the rapid filtration of the serum mixture depends upon the success of this conversion. Had there been any such change in the amount of protein remaining in solution in the serum mixture, there should have been corresponding variations in the refractive indices of the solution. But, as no change occurred in this physical property of the mixtures one must conclude that a certain amount of euglobulin is thrown out of solution on the addition of the ammonium sulphate at room temperature, and that this amount is not further increased during the heating process.

Since the conversion of pseudoglobulin to euglobulin apparently does not occur during the heating process, it is not improbable that the value of the heating lies in its complete aggregation of the particles of precipitated euglobulin into conglomerates of such dimensions that they are readily retained on the surface of filter paper. The advantage of rapid over slow preliminary heating of the serum mixture—*i.e.* up to 57°—in producing an easy filtering fluid, is most readily explained on the same ground. If the heating were insufficient the smallest particles would pass the filter, while the slightly larger would actually engage and block the pores, producing an ultra filter which would impede or even stop filtration.

With the object of testing the correctness of these hypotheses, the following experiments were accordingly instituted.

Experiment I. Separate volumes of plasma, undiluted or diluted, were made 20, ... 54 per cent. of saturation with ammonium sulphate. The refractive indices of these mixtures were measured and the protein still remaining in solution after the addition of the various amounts of ammonium sulphate solution was calculated, the results, for the purposes of comparison, being expressed in terms of the original plasma.

The various mixtures were then heated to a temperature of 61° or 63° according to the directions given in Banzhaf's paper, and the protein contents of the filtrates from samples of the mixtures were measured at the end of the heating process. The results have been embodied in Tables II, III, IV, and V and in Figure 1.

(*a*) The curve plotted in Figure 1, giving the relation between the concentration of ammonium sulphate in the mixtures and the amount of protein remaining in solution, shows that precipitation occurs mainly at percentages of saturation with ammonium sulphate greater than 28. However the gradient of the curve indicates no critical points for the precipitation of the eu- or pseudo-globulin fraction or of the serum albumins.

(*b*) After heating the mixtures containing concentrations of ammonium sulphate of less than 28 per cent. of saturation, clear liquids could not be obtained—the filtrates remained permanently opalescent and even on long standing the particles of the suspension did not coagulate sufficiently to be retained by filter paper.

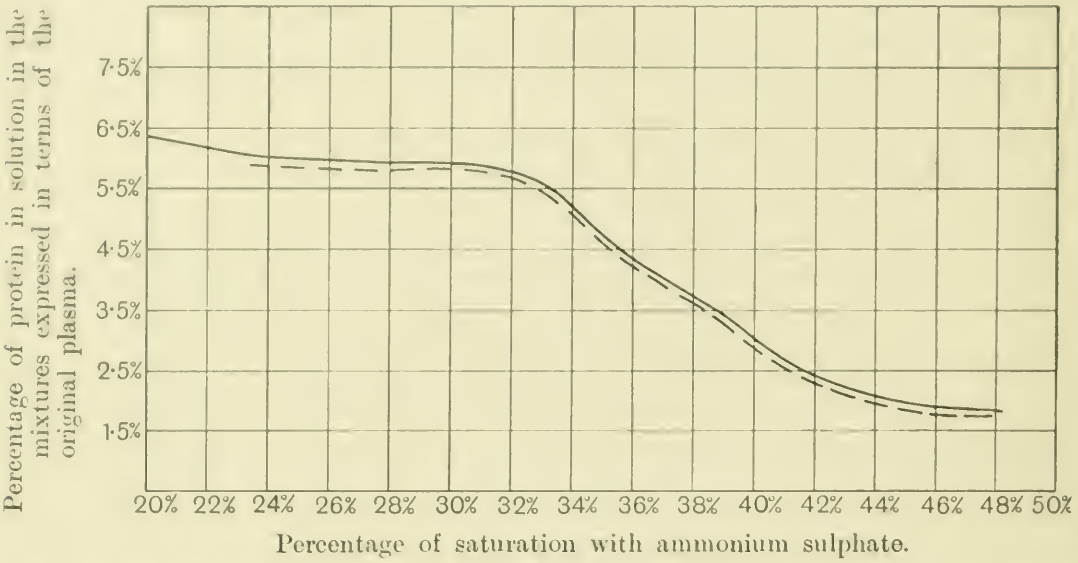

Fig. 1. Curves showing changes induced in the solubility of the proteins in serum mixtures made various percentages of saturation with ammonium sulphate during the process of heating the mixtures just to a temperature of 61° and keeping them at that temperature for a few minutes only.

The curve ——— represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ at room temperature.

The curve — — represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ and heated just to 61°.

(*c*) If the mixtures were kept at a temperature of 61° or 63° for a few minutes only, as advocated by Banzhaf, there was, during the heating process, no appreciable change in the protein content of the solution indicated by the refractometer (Tables II and III).

(*d*) If the mixtures were kept at a temperature of 61° or 63° for three-quarters of an hour, or longer (Table IV), then there was, during the heating process, a considerable change in the amount of protein in solution.

(*e*) If *undiluted* plasma were made various percentages of saturation with ammonium sulphate and the mixtures heated to 63° and kept at that temperature for a few minutes only, there was no appreciable change in the protein content of the solutions during the heating process (Table V).

TABLE II.

Oxalated plasma was diluted with half its volume of water and made various percentages of saturation with ammonium sulphate. The mixtures were then heated to a temperature of 61° and kept at that temperature for a few minutes, the heating process taking two hours in all.

Percentage of saturation with ammonium sulphate	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma[1]		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	6·49		
20	6·30	6·10	3·2
24	5·91	5·80	1·9
26	5·89	5·79	1·6
28	5·84	5·61	4·0
30	5·85	5·76	1·6
31	5·53	5·36	3·1
32	5·18	5·12	1·1
33	5·18	5·04	2·7
34	4·78	4·69	1·9
36	4·25	4·12	3·1
38	3·84	3·74	2·6
40	2·80	2·86	−2·1
44	2·02	1·90	6
48	1·90	1·90	0

TABLE III.

Oxalated plasma was diluted with half its volume of water and made various percentages of saturation with ammonium sulphate. The mixtures were heated to a temperature of 63° and kept at that temperature for a few minutes.

Percentage of saturation with ammonium sulphate	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	8·36		
25	7·35	(Too opalescent to be read)	
28	7·33	6·98	5·0
29	6·81	6·52	4·3
30	6·08	5·92	2·7
32	5·92	5·61	5·3
34	5·48	5·44	0·74
35	5·24	5·11	2·5
40	4·83	4·68	3·1
46	3·39	3·31	2·4
48	2·08	2·14	−3
50	1·31	1·31	0

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[1] Throughout these tables it has not been deemed necessary to make corrections for the fact that the addition of x cc. of ammonium sulphate solution to $100 - x$ cc. of serum does not necessarily yield a volume of 100 cc. of mixture.

TABLE IV.

Oxalated plasma diluted with half its volume of water was made various percentages of saturation with ammonium sulphate. The mixtures were heated to 61° or 63° and kept there for three-quarters of an hour.

	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma			Percentage conversion of *soluble* into *insoluble* protein during the heating process in periods	
Percentage of saturation with $(NH_4)_2SO_4$	(*a*) Before heating	(*b*) After heating just to 61°	(*c*) After 0·75 hr further heating at 61° or 63°	(*b*)	(*c*)
Original plasma	6·49				
30	5·76	5·63	4·28[1]	2·3	25·7
33	5·19	5·02	3·78[2]	3·3	27·2
34	4·81	4·67	3·55[3]	3·1	26·1

[1] Heated to 61° for 1 hour.
[2] Heated to 62° for 0·75 hour.
[3] Heated to 63° for 0·75 hour.

TABLE V.

Undiluted oxalated plasma was made various percentages of saturation with ammonium sulphate. The mixtures were heated to a temperature of 63° *and kept at that temperature for a few minutes only.*

	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma		
Percentage of saturation with $(NH_4)_2SO_4$	(*a*) Before heating	(*b*) After heating	Percentage conversion of *soluble* into *insoluble* protein during the heating process
Original plasma	7·14		
28	6·23	5·97	4·2
30	5·98	5·83	2·4
31	5·73	5·43	5·3
32	5·33	4·98	6·6
33	5·17	4·90	5·3

Experiment II. That there was no appreciable change in the pseudoglobulin content of the mixtures during the process of heating was demonstrated gravimetrically as follows.

Several volumes of plasma, both diluted and undiluted, were taken and to them was added respectively ammonium sulphate solution in quantity to make a series of percentage solutions of varying concentration. Each series was duplicated. The same process was carried out with serum.

Of the duplicates of each mixture the one was dialysed without heating and the other previous to dialysis was heated to a temperature of 61° or 63°, and kept there for a few minutes only. After dialysis the residues were made up to a certain volume, aliquot parts of the clear liquid were taken and gravimetric estimations were made on the protein contained therein. Two protein estimations were made on each dialysis residue:

(*a*) The total protein present soluble in saturated brine;

(*b*) The total amount of protein present not precipitated by 50 per cent. saturated ammonium sulphate.

In some cases the total protein in solution in the dialysis residues was estimated gravimetrically and also by means of the refractometer. Gravimetric estimations were invariably slightly higher than those obtained from the refractometer readings, thus indicating that in the dialysis residues there is, in colloidal suspension, a certain amount of insoluble euglobulin, the particles of which are too small to be retained by filtration through paper.

The gravimetric estimations, collected in Table VI, confirm the evidence obtained from the use of the refractometer that there has been no appreciable conversion of pseudoglobulin into euglobulin during the heating process.

The evidence thus afforded in Experiments I and II indicates that Banzhaf's supposition that the heating process in his new method serves to convert some of the pseudoglobulin into euglobulin does not provide such an explanation of the changes taking place as can be verified by quantitative examination.

At first sight it is difficult to reconcile the conclusions thus to be drawn from a study of these data with the obvious fact that, during the heating process of the serum mixture the bulk of the precipitate visibly increases. The following experiment, however, serves to throw light on this apparent paradox.

Experiment III. Serum mixtures were made from 25 to 54 per cent. of saturation with ammonium sulphate and, after standing at room temperature in stoppered flasks for 2 or 3 hours, were filtered. The clear filtrate was divided into two portions and kept in stoppered flasks. In the one portion, which stood at room temperature for several days, the clear liquid, within a few hours, became cloudy, and there was then a deposition of solid protein which gradually increased in bulk. The other portion was heated to 63° (two and a half hours being taken just to reach that temperature) and in every case a precipitate was formed, although at dilutions below 28 per cent. of saturation with ammonium sulphate the precipitate was so

TABLE VI.

Gravimetric estimation of the protein "soluble in saturated brine" in solution in the serum mixtures before and after being subjected to the heating process.

Volume of plasma or serum previously diluted with half its volume of water	Percentage protein content of original plasma	Electrolytes added	Treatment before dialysis	Proportion of mixtures taken for dialysis	Total protein soluble in saturated brine solution		Protein not precipitated by making dialysis residue 50 % saturation with $(NH_4)_2SO_4$	
					(*a*) in the dialysis residue	(*b*) in 100 cc. of original plasma	(*a*) in the dialysis residue	(*b*) in 100 cc. of original plasma
					g.	g.	g.	g.
A. PLASMA (oxalated)	7·14							
(i) 50 cc.	—	made 30 % of saturation with $(NH_4)_2SO_4$	unheated	whole = ppte + liquid	1·324	3·972	0·650	1·950
(ii) 50 cc.	—	,,	heated	whole	1·325	3·975	0·649	2·060
B. PLASMA (oxalated)	7·14							
(i) 50 cc.	—	made 32 % of saturation with $(NH_4)_2SO_4$	unheated	,,	1·424	4·272	0·635	1·905
(ii) 50 cc.	—	,,	heated	,,	1·576	4·728	0·6365	1·910
C. PLASMA (oxalated)	5·76							
(i) 50 cc.	—	,,	unheated	,,	0·995	2·984	0·602	1·806
(ii) 50 cc.	—	,,	heated	,,	1·015	3·045	0·602	1·805
D. SERUM (carbolised)	5·51							
(i) 50 cc.	—	,,	unheated	,,	0·678	2·034	—	—
(ii) 50 cc.	—	,,	heated	,,	0·600	1·800	—	—

finely divided that the liquid could not be filtered clear for refractometer readings. In all cases, whether the filtrates had been heated or not, although there was the visible formation of solid matter from the previously clear filtrate, there was no change in the refractive indices of the solutions, thus indicating no further change in the protein content of the solution after the immediate effect due to the addition of the electrolyte at room temperature. Moreover, this delayed, slowly forming, precipitate was not a special feature of the mixtures containing a percentage of saturation with ammonium sulphate insufficient to precipitate completely the euglobulin from solution, *i.e.* up to 33 per cent. of saturation with ammonium sulphate, for it occurred in all of the ammonium sulphate-serum mixtures.

We are therefore led to no other conclusion than that the effect of heating the serum mixtures just to 61° or 63° merely serves to accelerate a change which takes place slowly at ordinary room temperature, and that whatever this change may be, it is not concerned with the conversion of the pseudoglobulin into the euglobulin.

Light may however be thrown on the question by a consideration of the following observations. Proteins belong to the hydrophile type of colloids, and in solution form an emulsoid system. It has been demonstrated by Chick and Martin [1913] and others that if a high concentration of electrolytes be added to a solution of a protein, there is withdrawal of water from the internal phase of the emulsoid system, and if the withdrawal be sufficient, this phase becomes rigid and is eventually precipitated. In the salting out of proteins the emulsoid is thus first converted into a suspensoid system. Applying these observations to the salting out of serum proteins, it is probable that in the fractional precipitation, with additions of ammonium sulphate to less than 33 per cent. of saturation, the euglobulin is first changed from the emulsoid to the suspensoid type of colloid: the suspensoid is in part converted into a suspension and the latter settles as a precipitate. The protein thus converted into a suspensoid or suspension will no longer have a marked effect on the viscosity or on the refractive index of the solution—even though the particles be neither visible to the naked eye, nor retained by filter paper.

The experimental evidence given above (Experiment III) shows that the complete agglutination of the particles of the suspensoid to those of a perceptible precipitate takes place slowly at ordinary temperatures, and that the effect of heating the liquids in the new process merely serves to increase the velocity of this change. Clearly then, the change thus taking place is not that suggested by Banzhaf, viz. the conversion of pseudoglobulin into euglobulin, but rather a more simple physical process involving the agglutination of the particles of euglobulin already thrown out of emulsoid suspension by the ammonium sulphate. This agglutination permits of easy separation of the euglobulin by filtration through paper.

If, for some reason or other, in the routine concentrations of batches of sera or plasma, the precipitated euglobulin is not completely agglutinated during the heating process, then difficulties will be encountered in the subsequent process of filtration. Some of the insufficiently agglutinated aggregates readily pass through the pores of the filter paper, while others, not sufficiently large to be retained on the surface of the paper, either partially or wholly block the pores of the paper and so retard the process of filtration. (Table VII shows that the partially blocked paper may act as an ultra filter.) The euglobulin thus passing into the filtrate will be precipitated with the pseudoglobulin and antitoxin at one-half saturation with ammonium sulphate. Theoretically speaking, when the precipitated pseudoglobulin and antitoxin

TABLE VII.

Experiment to demonstrate the power of insufficiently agglutinated particles in a badly filtering heated serum mixture to convert an ordinary filter paper into an ultra filter.

Oxalated plasma, diluted with half its volume of water and made various percentages of saturation with ammonium sulphate had been heated just to 61°. The serum mixtures filtered badly; at first there was an opalescent filtrate but the filtration soon slackened and almost ceased.

The protein content of the first opalescent filtrate was estimated and compared with that of the slowly forming clear drops of filtrate collected a short time before the filtration ceased.

	Residual percentage of protein in solution calculated from the refractometer readings of the filtrates of the mixtures and expressed, for purposes of comparison, in terms of the original plasma			
	(*a*) Before heating	(*b*) After heating		The self made ultra filter has effected the removal of soluble protein to the extent of
Percentage of saturation with $(NH_4)_2SO_4$		(i) First filtrate slightly opalescent	(ii) Clear drops just before filtration ceased	
Original plasma	6·49			
24	5·91	5·80	4·68	20·8 %
26	5·89	5·80	4·69	20·3 %
28	5·84	5·61	4·86	16·8 %
30	5·85	5·76	5·30	9·5 %
31	5·53	5·36	5·22	5·6 %

containing a small amount of euglobulin is dialysed until the salt content approaches that of tap water, the pseudoglobulin and antitoxin only should be in solution, but it is generally accepted that the presence of one protein in solution protects another from precipitation.

We have seen (Experiment II) that even in dilute dialysis residues a small amount of euglobulin remained in colloidal suspension. Since, in routine concentrations, the dialysis residues may contain up to and even more than 20 per cent. of pseudoglobulin, the protecting power of the latter in respect of the euglobulin is enhanced. The euglobulin thus in colloidal suspension will greatly retard, if not render impossible, the filtration of the final product through filter candles.

The impediment to filtration in the new process caused by the suspensoid particles of euglobulin was in practice removed by allowing the dialysis residue to stand for a prolonged period. This tedious method has had to be resorted to frequently.

II. *An investigation of the rôle of Sodium Chloride in improving the Banzhaf new One Fraction Method.*

Having ascertained the value of the heating process in the new method, the question then arose as to the part played by the sodium chloride in improving the concentration process. Did the addition of salt to the serum mixtures serve to induce the desired conversion of pseudoglobulin into euglobulin or did it merely exert some stimulating effect on the agglutination of the aggregates of the euglobulin, a process which, from the experimental work in Part I, seems to take place slowly?

In order to elucidate this point, routine concentrations in which 1·5 per cent. of solid sodium chloride had been added to serum or plasma mixtures which had been made 30 per cent. or 31 per cent. of saturation with ammonium sulphate were carefully examined at different stages of the heating process. As before, samples were taken from the mixtures and filtered, and the protein content of the clear filtrates was estimated from the difference between the refractometer readings of the clear filtrate before and after coagulation of the protein therein. For purposes of comparison the protein content of the mixtures at various stages in the heating process was expressed in terms of the original serum or plasma.

Up to the present the concentration of 20 batches each of not less than 50 litres of plasma or serum has been followed; the data with regard to three typical concentrations are given in Table VIII. In no case has any difficulty been experienced in the filtration of the hot liquids or of the final product so far as this latter has up to the present been filtered. In no case has there been any appreciable change in the protein content of the mixtures during the heating process.

From these observations we are led to the conclusion that the effect of the heating process is to agglutinate the particles of euglobulin already converted from the emulsoid to the suspensoid state. This process, which seems to be incomplete in some serum mixtures, is stimulated by the addition of sodium chloride to the same mixtures.

TABLE VIII.

Changes taking place during the heating process to 63° *or* 65° *in the protein content of routine concentration serum mixtures which have been made* 30 *or* 31 *per cent. of saturation with ammonium sulphate and* 1·5 *per cent. with solid sodium chloride.*

Amount of oxalated plasma concentrated	Protein content of original plasma	Percentage of saturation with ammonium sulphate	Duration of time of heating of mixtures	Temperature of mixtures at which samples were taken	Residual percentage of protein in solution in the filtrates of the serum mixtures, calculated from the refractometer readings and expressed in terms of the original plasma
Diphtheria 50 litres	7·00	31	0	Room. temp.	5·11
			2 hrs	57°	5·00
			2 hrs 35′	61°	5·02
			3 hrs	63°	4·93
Tetanus 50 litres	7·47	30	1 hr 55′	57°	6·82
			2 hrs 25′	61°	6·60
			2 hrs 55′	63°	6·60
Tetanus 50 litres	6·73	30	0	Room temp. = 16°	5·19
			1 hr 35′	57°	5·28
			2 hrs 15′	61°	5·28
			2 hrs 35′	65°	5·12

Additional evidence on this point was furnished by the following experiments.

Experiment IV. Serum or plasma (diluted or undiluted) was made from 25–50 per cent. of saturation with ammonium sulphate and solid sodium chloride was added to the mixture to the extent of 1·5 per cent. The protein content of the filtrates of samples of the mixtures was estimated from the refractometer readings as in the previous experiments. The mixtures were then subjected to various treatments.

(*a*) The mixtures were heated to 63° and kept at that temperature for a few minutes, the process in all taking from 2 to 2·5 hours. (Table IX.)

It will be seen from the measurements of the refractive indices of the solutions and from the curves in Figure 2 that there was no appreciable change in the pseudoglobulin content of the mixtures during the process of heating.

(*b*) The mixtures were heated to a temperature of 67° and kept at that temperature for 10 minutes. (Table X and Figure 3.)

TABLE IX.

Oxalated plasma diluted with half its volume of water, made various percentages of saturation with ammonium sulphate and 1·5 *per cent. solid sodium chloride added to the mixtures. The mixtures were heated to a temperature of* 63° *and kept at that temperature for a few minutes.*

Percentage of saturation with ammonium sulphate	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates from the mixtures and expressed, for purposes of comparison, in terms of the original undiluted plasma		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	8·30		
29	6·93	6·66	4·0
30	6·73	6·52	3·1
31	6·38	6·14	3·8
32	5·97	5·89	1·36
33	5·09	4·85	0·8
34	4·81	4·70	2·96
36	3·73	3·64	2·5
39	2·44	2·39	2·1
40	2·19	2·19	0
46	2·15	2·15	0
54	0·574	0·582	– 1·6
Original plasma	7·14		
29	5·68	5·44	4·3
30	5·30	5·25	1
31	5·12	5·08	0·8

From the results it is clear that heating the serum mixtures to this higher temperature is accompanied by a considerable change in the protein content of the solutions.

(*c*) The mixtures were heated to a temperature of 63° and kept at that temperature for 1·25 hours. (Table XI and Figure 3.)

Obviously the prolongation of the time of heating at 63° also gives rise to considerable change in the protein content of the solution.

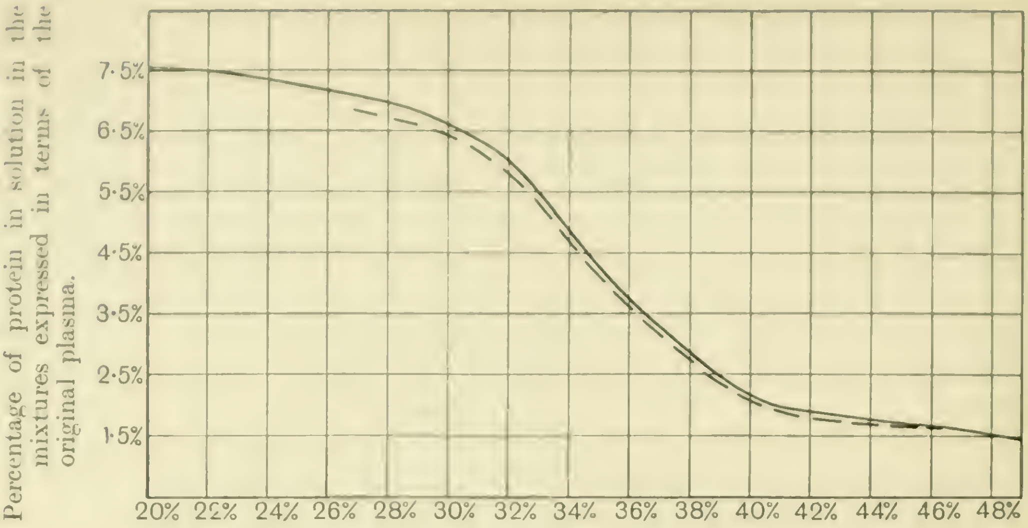

Fig. 2. Curves showing changes induced in the solubility of the proteins in serum mixtures made various percentages of saturation with ammonium sulphate and 1·5 per cent. with solid sodium chloride during the process of heating the mixtures just to a temperature of 63° and keeping them at that temperature for a few minutes only.

The curve ——— represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ at room temperature.

The curve — — — represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ heated to 63° and kept at that temperature for a few minutes only.

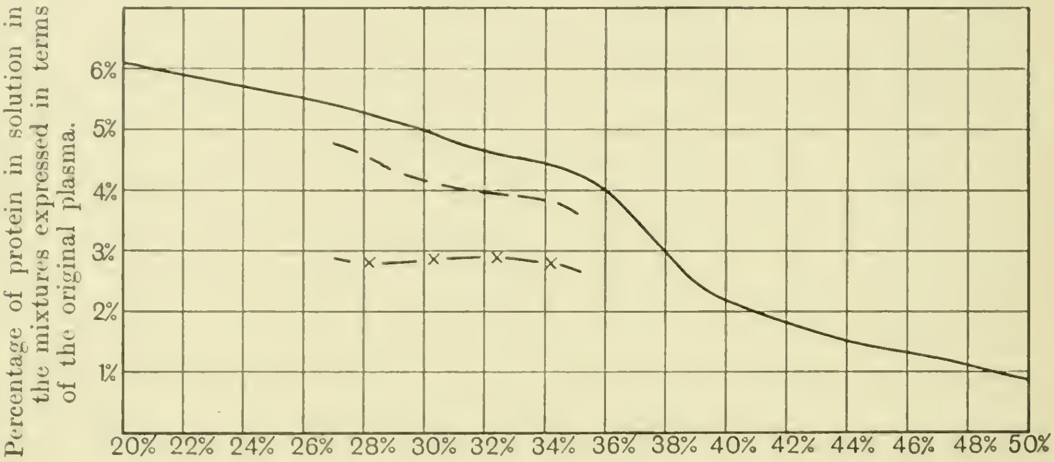

Fig. 3. Curves showing changes induced in the solubility of the proteins in serum mixtures made various percentages of saturation with ammonium sulphate, during the process of heating (*a*) just to a temperature of 67° and (*b*) to a temperature of 63° and keeping at that temperature for 1¼ hours.

The curve ——— represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ at room temperature.

The curve — — — represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ and heated just to 67°.

The curve — × — × — represents the amount of protein remaining in solution in the mixtures made various percentages of saturation with $(NH_4)_2SO_4$ and heated to 63° and kept at that temperature for 1¼ hours.

Experiment V. That there was no appreciable change in the pseudoglobulin content of the mixtures during the heating process adopted in the new method was also demonstrated gravimetrically.

Several volumes of serum or plasma, diluted or undiluted, were respectively made various percentages of saturation with ammonium sulphate, and 1·5 per cent. of solid sodium chloride was then added. Each series was duplicated and of the duplicates the one was dialysed without heating and the other, previous to dialysis, was heated to a temperature of 63° and kept at that temperature for a few minutes only—the heating process taking 2 to 2·5 hours in all.

TABLE X.

Oxalated plasma diluted with half its volume of water was made various percentages of saturation with ammonium sulphate and 1·5 *per cent. of solid sodium chloride was added to the mixtures which were then heated to a temperature of* 67°.

Percentage of saturation with $(NH_4)_2SO_4$	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates from the mixtures and expressed, for purposes of comparison, in terms of the original plasma		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	6·23		
28	5·34	4·66	12·9
29	5·14	4·24	17·7
30	4·98	4·00	20·0
31	4·97	4·25	14·5
32	4·62	3·99	13·6
33	4·54	4·05	11.0
34	4·56	3·96	10·4

The protein estimations were made on each dialysis residue, viz.:

(*a*) The total protein present soluble in saturated brine solution.

(*b*) The total amount of protein not precipitated by 50 per cent. of saturation with ammonium sulphate.

The results showed that, during the heating process, there was no appreciable conversion of pseudoglobulin into euglobulin.

From the results given in Tables VI and XII it is interesting to note that the amount of protein precipitated from the dialysis residues by saturation with brine depends upon the original dilution of the serum or plasma. This is a generally accepted fact (Banzhaf-Gibson).

In certain cases the whole serum mixture, *i.e.* precipitate and liquid, as well as controls of the filtrate alone were dialysed, when it was observed that the pseudoglobulin was much greater in amount in the former than in the latter case: that is to say that the precipitate brought down by the ammonium sulphate was in reality a mixture of eu- and pseudo-globulin.

Further, in those instances in the above experiment in which oxalated plasma was used, the dialysis residues of the unheated plasma mixtures contained the insoluble fibrin and euglobulin formed into a jelly-like clot. In the dialysis residues of the heated mixtures these insoluble proteins were present as a flocculent and readily subsiding precipitate.

Experiment VI. Further experimental work was then undertaken to ascertain the effect of sodium chloride on the solubility of serum proteins under different conditions.

TABLE XI.

Oxalated plasma, diluted with half its volume of water, was made various percentages of saturation with ammonium sulphate and 1·5 *per cent. solid sodium chloride added to the mixtures which were then heated to* 63° *and kept at that temperature for* 1·25 *hours.*

Percentage of saturation with $(NH_4)_2SO_4$	Residual percentage of protein in solution, calculated from the refractometer readings of the filtrates from the mixtures, and expressed, for purposes of comparison, in terms of the original plasma		Percentage conversion of *soluble* into *insoluble* protein during the heating process
	(*a*) Before heating	(*b*) After heating	
Original plasma	6·23		
28	5·44	2·81	48·6
30	5·29	2·89	45·5
32	4·56	2·80	39·0
34	4·62	2·86	38·4

(*a*) *The effect of heating a saturated brine solution of pseudoglobulin.*

The globulin precipitate thrown down by making plasma 50 per cent. of saturation with ammonium sulphate was suspended in saturated brine solution and after standing at room temperature for 48 hours was gradually heated to a temperature of 61°, the process taking 2 hours in all.

From the use of the refractometer it was ascertained that at room temperature (after 48 hours) the saturated brine contained protein in solution corresponding with a value of 6·3 per cent. in the original plasma. After

TABLE XII.

Gravimetric estimation of the protein "soluble in saturated brine" present in serum mixtures made various percentages of saturation with ammonium sulphate and 1·5 per cent. with sodium chloride, before and after being heated to 63° and kept at that temperature for a few minutes only.

	Percentage protein content of citrated plasma taken	Dilution of plasma	Volume of diluted plasma taken	Percentage of saturation with $(NH_4)_2SO_4$	Treatment of mixtures *previous to dialysis*	Total protein soluble in saturated brine		Protein not precipitated at 50 per cent. of saturation with $(NH_4)_2SO_4$	
						(*a*) the dialysis residue	(*b*) 100 cc. of original serum or plasma	(*a*) in the dialysis residue	(*b*) in 100 cc. of original plasma or serum
A.	7·14	undiluted	100 cc.	29	The mixture, after standing 2 hrs, was filtered. Of the clear filtrate				
					(i) 50 cc. were unheated	1·074	3·024	0·633	1·880
					(ii) 50 cc. were heated	1·098	3·091	0·620	1·745
B.	7·14	undiluted	100 cc.	31	The mixture, after standing 2 hrs, was filtered and of the clear filtrate				
					(i) 50 cc. were unheated	1·596	4·62	0·624	1·800
					(ii) 50 cc. were heated	1·700	4·92	0·600	1·714
C.	7·14	undiluted	2 lots of 50 cc. each	31	The whole mixtures, *i.e.* ppte and liquid, were respectively taken and				
					(i) unheated	2·68	5·36	1·17	2·34
					(ii) heated	2·56	5·12	1·13	2·26
D.	5·76	diluted with ½ its vol. of H_2O	100 cc.	31	The mixture, after standing 2 hrs. was filtered and of the clear filtrate				
					(i) 50 cc. were unheated	0·234	0·641	—	—
					(ii) 50 cc. were heated	0·227	0·621	—	—

raising the temperature of the brine solution just to 61°, the protein in solution had decreased to the value of 2·48 per cent.

During the heating process there had thus been a conversion of 60·6 per cent. of the soluble into insoluble protein; at the same time there was considerable loss of antitoxic units from the solution.

TABLE XIII.

The effect of the prolonged heating of plasma, diluted with one-third its volume of water and containing 1·5 *per cent. of sodium chloride, upon* (*a*) *the solubility of the serum proteins and* (*b*) *the precipitating power of* 30 *per cent. of saturation with ammonium sulphate.*

		Residual percentage of protein in solution		
Length of time diluted plasma or serum was heated	Temperature of heated liquids	(*a*) in the diluted plasma or serum at various stages in the heating process calculated from the refractometer readings of the heated liquids and expressed, for purposes of comparison, in terms of the original plasma	(*b*) after making the heated liquids 30 per cent. of saturation with $(NH_4)_2SO_4$, calculated as in (*a*)	Percentage increase in the amount of soluble protein precipitated by 30 per cent. of saturation with $(NH_4)_2SO_4$
Plasma (*oxalated*)				
0 hrs	Room temperature	7·38	5·12	—
3 hrs	57°	7·05		
7 hrs	57°	7·01	4·84	5·5
Serum (*carbolised*)				
(1) 0 hrs	Room temperature	6·47	5·82	
15 hrs	57°	5·99	3·81	34·6
(2) 0 hrs	Room temperature	6·95	5·41	
15 hrs	57°	5·77	3·13	42·2

(*b*) *Prolonged heating of serum or plasma to which* 1·5 *per cent. sodium chloride had been added.*

Plasma and serum were each diluted with one-third of their volume of water, 1·5 per cent. of salt added, and heated to a temperature of 57° for periods varying from 6 to 15 hours.

The refractometer readings showed that during the heating process there was a slight change in the protein content of the solutions, but this change did not take place with less than three hours' heating. Samples of the heated mixtures, taken after 6 and 15 hours' heating respectively, were made 30 per cent. of saturation with ammonium sulphate and the protein thrown out of solution compared with that precipitated by the same saturation with ammonium sulphate in the original unheated serum or plasma. (Table XIII.)

The results show that the amount of protein remaining in solution after making the serum 30 per cent. of saturation with ammonium sulphate is practically the same for the unheated serum as for the serum heated to 57° for 6 hours. After 15 hours' heating there is a 30–40 per cent. increase in the precipitation of protein.

Further heating of the respective ammonium sulphate mixtures to a temperature of 61° was not accompanied by any increased change in the protein content of the solutions.

(c) *The heating of serum or plasma without the addition of salt.*

In order to ascertain whether the change thus taking place in (*b*) during the prolonged heating of plasma or serum with 1·5 per cent. sodium chloride was due to the specific action of the sodium chloride on the proteins of horse serum or was an inherent property of the proteins themselves, plasma and serum diluted with water were heated, *without the addition of salt*, in order to ascertain whether changes had been induced during the heating.

(α) *Changes in the solubility of the serum proteins.*

During the heating process there was slight deposition of proteins and samples of the heated liquids filtered with difficulty.

From Table XIV it will be seen that whether the serum or plasma had been heated at 63° for a short time or kept at lower temperatures for longer periods there was a slight change in the solubility of the proteins.

(β) *Changes in the precipitating power of* 30 *per cent. of saturation with ammonium sulphate.*

The results depicted in Table XV show that considerable changes may take place in the precipitating power of this reagent.

It has been previously demonstrated that the heating of the serum to 63°, when the liquid was kept at that temperature for a few minutes only, was not followed by any appreciable change in the amount of protein precipitated by 30 per cent. of saturation with ammonium sulphate. But if the liquids were kept at this temperature for 1 hour, or if the temperature were raised to 67°, then considerably more protein was precipitated (from 10 to 20 per cent. increase).

TABLE XIV.

The effect of the heating of serum or plasma on the solubility of the serum proteins [*No addition of* NaCl.]

Dilution of the oxalated plasma or serum	Temperature to which heated	Duration of the time of heating at the specified temperature	Percentage of protein in solution in the plasma—measured by means of the refractometer		Percentage conversion of *soluble* into *insoluble* protein during the heating process
			(*a*) Before heating	(*b*) After heating	
(1) *Plasma* with $\frac{1}{2}$ its vol. of H_2O	63°	10 min.	7·69	7·43	3·4
(2) *Plasma* with $\frac{1}{3}$ its vol. of water	57°	0 hrs	5·85	—	
	57°	15 hrs	5·85	5·30	9·4
(3) *Plasma* with $\frac{1}{3}$ its vol. of water	57°	0 hrs	6·14	—	
	57°	4 hrs	,,	5·99	2·4
	57°	15 hrs	,,	5·79	5·7
(4) *Carbolised serum*[1] with $\frac{1}{3}$ its vol. of water	57°	0 hrs	5·59	—	
	57°	15 hrs	,,	3·79	32·2

[1] A considerable change always takes place during the heating of carbolised serum. This point is being investigated.

TABLE XV.

The effect of the prolonged heating of plasma and of serum respectively diluted with one third their volume of water upon the precipitating power of 30 *per cent. of saturation with ammonium sulphate.* [*No addition of* NaCl.]

Temperature reached	Duration of the heating at the specified temperature	Percentage of protein in solution at the stage in the heating process. Expressed in terms of the original plasma	Percentage of protein remaining in solution after making the liquid 30 per cent. of saturation with ammonium sulphate. Expressed in terms of the original plasma	Percentage change in the amount of dissolved protein precipitated by 30 per cent. of saturation with $(NH_4)_2SO_4$
Carbolised serum				
57°	0 hrs	5·59	4·53	
	15 hrs	3·79	2·42	46·0
Plasma				
57°	0 hrs	5·85	5·80	
	15 hrs	5·30	3·52	39·3
Plasma				
57°	0 hrs	6·14	5·78	
	4 hrs	5·99	5·48	5·2
	15 hrs	5·79	3·52	39·0
Plasma				
55°	0 hrs	7·39	5·35	
	15 hrs	6·89	4·81	10·0

If the plasma or serum were kept at a temperature of 55° for 15 hours there was a 10 per cent. increase in the precipitating power of 30 per cent. saturation with ammonium sulphate.

When the plasma or serum had been kept at 57° for 4 hours there was very little difference between the amounts of protein precipitated from the heated and unheated liquids by the addition of saturated ammonium sulphate to the extent of 30 per cent. of saturation.

After 15 hours' heating at 57° it was found that, on the addition of ammonium sulphate to 30 per cent. of saturation, from 39–47 per cent. more of the soluble protein was precipitated from the heated than from the unheated liquids.

It is thus obvious that the change taking place in the prolonged heating of serum or plasma with 1·5 per cent. sodium chloride in (*b*) is in no way dependent on the presence of the sodium chloride, as the same phenomenon takes place in the prolonged heating of serum or plasma to which no salt has been added.

That changes take place in the prolonged heating of solutions of pseudoglobulin has been shown by Banzhaf [1908] but from the details given in his paper it is difficult to understand the exact procedure adopted by him. Banzhaf suggests that the change is due to the conversion of pseudoglobulin into euglobulin, but from consideration of the work of Hardy [1899], Chick and Martin [1912], and Chick [1914] it is probable that the change taking place in (*b*) and (*c*) above is of the nature of a denaturation of proteins. For, evidently, during the prolonged heating of the serum or plasma, while the actual solubility of the proteins has been little changed their physical properties have become so altered that their precipitability by electrolytes is considerably increased.

In view of these observations it is at first sight not easy to picture the part played by the sodium chloride in rendering the concentration of antitoxic sera by Banzhaf's new process more certain of success. If it were merely a question of the better salting out of the euglobulin by increasing the dehydrating action exerted by the salt, then why should the addition of 1·5 per cent. sodium chloride to the 29 or 30 per cent. of saturation with ammonium sulphate serum mixtures yield a better and more easily filterable end product than that obtained from 31, 32, 33 per cent. or even 34 per cent. of saturation in the absence of sodium chloride.

Now Banzhaf and Gibson [1907] have stated that the salting out of euglobulin is influenced by the dilution of the plasma and they have given what is, in their opinion, the optimum dilution for routine concentrations.

But, that factors other than the dehydrating effect of the salts added to the serum and the dilution of the serum proteins have to be considered, is shown by an examination of the data collected during the progress of this research. (Table XVI.)

TABLE XVI.

The percentage of protein remaining in solution in serum mixtures made 30 or 31 per cent. of saturation with ammonium sulphate.

Percentage protein content of the original plasma taken	Treatment of the diluted plasma		Residual percentage of protein remaining in solution in the mixtures and expressed in terms of the original plasma
	(*a*) Percentage of saturation with $(NH_4)_2SO_4$	(*b*) Percentage of NaCl in the mixtures	
7·14	30	—	4·93
6·47	,,	—	5·85
6·47	31	—	5·53
8·10	,,	—	6·29
8·36	,,	—	6·39
7·14	,,	—	5·74
6·71	30	1·5	5·16
6·73	,,	,,	4·19
7·38	,,	,,	4·97
7·14	,,	,,	5·20
7·38	,,	,,	5·12
6·38	,,	,,	5·81
5·51	,,	,,	4·53
5·76	,,	,,	5·80
6·04	,,	,,	5·76
6·23	,,	,,	5·29
8·32	,,	,,	6·39
8·32	31	1·5	5·97
7·14	,,	,,	5·12
7·28	,,	,,	5·11
7·00	,,	,,	5·11
6·71	,,	,,	4·61
7·47	,,	,,	6·80
7·28	,,	,,	4·42
6·79	,,	,,	5·59

It will be seen, that though the ammonium sulphate concentration and the protein concentration of some of the samples were practically identical, yet the amount of protein left in solution was considerably different. Evidently factors operated which had not been controlled.

It is believed that colloidal aggregates are kept in solution by their mutual repulsion which is due to the electric charge they carry. If this charge be sufficiently reduced, then the forces keeping the particles apart diminish; at the iso-electric point the colloids will be most completely precipitated. In other words, the more nearly the hydrogen-ion concentration of the system approaches that of the iso-electric point for a particular protein, the more complete will be the precipitation of that protein.

Therefore, when dealing with the concentration of antitoxic sera on a large scale, we ought not to lose sight of the fact that the reaction of the serum mixtures is probably of more vital importance in the salting out process than has hitherto been recognised in the methods of practical value advocated for the concentration of antitoxic sera. The more nearly the reaction of the serum mixtures approaches the hydrogen-ion concentration at the iso-electric point of euglobulin, not only should there be more rapid and effective filtration, but more constant results should be obtained with regard to the percentage removal of protein from the solution.

In the Banzhaf new One Fraction Method no cognisance is taken of the reaction of the mixture and this may well be the secret of its uncertainty.

The alkalinity of blood is supposed to be due to the partial hydrolysis of certain sodium salts of weak acids, and if this be the case the introduction of a salt like sodium chloride should slightly reduce the alkalinity. It therefore follows that the stimulating effect of the sodium chloride may be due to the fact that it tends to make the hydrogen-ion concentration of the mixtures approach more nearly that required for the optimum precipitation of euglobulin.

In support of this theory it may be mentioned that during the past few months I have had occasion to study the heat coagulation of 350 samples of serum. With some samples the heat coagulation could be readily brought about. With others the formation of a readily filterable coagulum could only be induced after the addition of from 1 to 6 per cent. of sodium chloride, or by the addition of corresponding amounts of a standard solution of acetic acid. The sodium chloride and the acetic acid were interchangeable and apparently had the same effect whether used separately or whether a certain proportion of the one was replaced by the corresponding proportion of the other.

In this connection experiments are being conducted with the object of ascertaining the influence of alteration of the reaction (hydrogen-ion concentration) of the serum mixtures on the precipitation and filtration of the eu- and pseudo-globulin and antitoxin fractions. For, although the desired improvement in filtration may be obtained by the addition of acetic acid to the more alkaline sera we must not lose sight of the fact that, as salt is known to exert a protecting action on antitoxin, it may be better to adjust the reaction of the medium by means of salt rather than by the addition of acid. The results will be dealt with in a future communication.

SUMMARY.

1. The advantages claimed by Banzhaf for his new One Fraction process are sometimes vitiated by the fact that a final product is obtained which, owing to the presence of an opalescent suspension presumably of euglobulin, cannot be filtered except after long standing.

2. The successful preparation of an easily filterable end product depends on the initial heating of the serum mixtures.

3. The heating process advocated in the new method does not cause a conversion of pseudoglobulin into euglobulin but merely serves to complete the aggregation of the euglobulin into particles sufficiently large to admit of separation by filtration.

4. The uncertainty with regard to the successful heating of the serum mixtures can be obviated by a slight modification of the described technique, viz. by the addition of 1·5 per cent. sodium chloride to the serum mixtures.

5. The following observations have been made with regard to the effect of the addition of sodium chloride to the serum mixtures:

(*a*) During the process of heating the mixtures to 63° or 65° the addition of salt does not induce changes in the amount of proteins still remaining in solution, but if the temperature be raised to 67° or if the mixtures be kept for one hour or more at 63°, then a considerable change in the protein content of the solution takes place.

(*b*) If a solution of pseudoglobulin and antitoxin in saturated brine be heated to a temperature of 61° and kept at that temperature for a few minutes only, there is a conversion of 60 per cent. of soluble into insoluble globulin, but at the same time there is loss of antitoxic units.

(*c*) Prolonged heating of serum or plasma diluted with water and containing 1·5 per cent. sodium chloride leads to an increase in the precipitating power of 30 per cent. of saturation with ammonium sulphate. This change, however, is not due to the presence of the sodium chloride. The prolonged heating of diluted plasma or serum gives rise to the same phenomenon.

Banzhaf considers that the change thus produced in the serum is that of pseudoglobulin into euglobulin, but it seems more probable that the prolonged heating serves to denaturate the protein in solution and to make it more readily precipitable by saturation with sodium chloride or by 30 per cent. saturation with ammonium sulphate.

6. It is possible that the addition of sodium chloride exerts a favourable influence on the filtration processes by virtue of an adjustment of the hydrogen-ion concentration of the serum mixtures.

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N° 6

AN IMPROVED METHOD FOR THE CONCENTRATION OF ANTITOXIC SERA.

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Gibson (1905) was the first to introduce a method of commercial value for the concentration of antitoxic horse sera.

He treated plasma with an equal volume of saturated ammonium sulphate solution, filtered off the precipitated globulins and suspended the latter in saturated salt solution. The pseudoglobulin and antitoxin entered into solution while the euglobulin remained in suspension. The solution of pseudoglobulin and antitoxin was freed from euglobulin by filtration and the clear filtrate was then acidified with acetic acid. The precipitated pseudoglobulin and antitoxin were dialysed. The finished product had a potency two and a half times greater than that of the original plasma.

In 1907 Banzhaf and Gibson, by fractional precipitation with ammonium sulphate, separated the pseudoglobulin-antitoxin combination from the other serum proteins as follows:

The plasma or serum, diluted with one-third its volume of water, was heated to a temperature of 57° C. for 15 hours or of 58° C. for 7 hours. The liquid was then made 30 % saturation with ammonium sulphate[1] and the ensuing precipitate (Fraction 1) separated by filtration.

The Fraction 1 precipitate was suspended in saturated brine. The brined liquid was filtered from the insoluble euglobulin and the filtrate, containing in solution the pseudoglobulin and antitoxin which had been present in the Fraction 1 precipitate, was made 0·25 % with glacial acetic acid. The ensuing precipitate was filtered, pressed and dialysed.

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[1] The phrase "made x % saturation with ammonium sulphate" has been used to indicate the following: to $100-x$ parts of the serum by volume there was added x parts by volume of a saturated aqueous solution of ammonium sulphate.

The filtrate from Fraction 1 was made 54 % of saturation with ammonium sulphate and the precipitate (Fraction 2) thus formed was filtered, pressed and dialysed. The residue from the dialysis of the second Fraction precipitate is the more potent and may be used separately. In practice the dialysis residues from Fractions 1 and 2 were usually mixed and the requisite amount of salt and preservative was added. The finished product by this method had a protein content of about 18 to 20 % and a unitage per c.c. about 4 to 5 times greater than that of the original plasma.

In 1912–13 Banzhaf introduced his One Fraction method in which serum or plasma, diluted with half its volume of water and made 30 % of saturation with ammonium sulphate, is brought up to a temperature of 60° C. and kept at that temperature for a few minutes only, the entire heating process taking about 2 hours in all.

The hot serum mixture is then filtered. The precipitate of euglobulin is well pressed or is washed with 33 % saturation with ammonium sulphate; the washings are filtered and added to the main bulk of the filtrate which is then made 50 % of saturation with ammonium sulphate. The ensuing precipitate, consisting of pseudoglobulin and antitoxin, is filtered, pressed and dialysed. The dialysis residues are treated in the same way as in the Banzhaf-Gibson process.

The protein content of the finished product is about 20 % while the unitage per c.c. is 4 to 5 times that of the original serum.

Banzhaf's new method certainly possesses the following advantages over the former methods:

(1) There is only one finished product for dialysis.

(2) There is a considerable saving of time as the lengthy process of extracting the first fraction precipitate with brine is obviated.

(3) The expenses incurred in the process are considerably less, both as regards labour and the use of precipitating materials, etc.

But unfortunately practical experience shows that the method is not always easy to work as the production of a readily filterable end product which will remain clear is dependent upon the condition of the serum mixtures after the preliminary heating process. If the latter do not filter rapidly and easily the end product will not be satisfactory. In such cases it is necessary to allow the dialysis residues to stand in the ice chest for weeks so that the opalescent suspension therein may settle, otherwise filtration through filter candles is accomplished with great difficulty and the formation of a solid deposit in the antitoxin in its vial containers occurs.

This delay is a great disadvantage and nullifies any gain in time which should accrue from the fact that there is only one fraction to be considered.

In this laboratory we have ascertained that the irregularities in the filtration can be obviated by the addition of 1½ to 2 % of solid sodium chloride to the serum mixtures thus:

The serum or plasma is diluted with one-half its volume of water, made 30 or 31 % of saturation with ammonium sulphate and to the mixture is added 1½ to 2 % of solid sodium chloride. The temperature of the mixture is gradually raised to 61° or 63° C. and kept at the chosen temperature for not more than five minutes. The mixture is cooled to 40°–45° C. before filtration.

The filtration of the hot mixture and of the subsequent 50 % saturation mixture proceeds rapidly. Moreover the end product has, up to the present, proved satisfactory.

Banzhaf considers that in his new process the value of the heating lies in the conversion of soluble pseudoglobulin into a further amount of euglobulin and that the rapid filtration of the serum mixture depends upon the success of the operation. In a recent communication (1916), however, I have demonstrated that there is no appreciable conversion of soluble into insoluble protein under the heating conditions advocated in his One Fraction process. The value of the heating process lies in the complete aggregation of the particles of precipitated euglobulin into conglomerates of such dimensions that they are readily retained on the surface of filter paper. The particles of euglobulin not sufficiently aggregated to be retained by the filter paper will ultimately be an impediment to the filtration of the final product through filter candles. Any suspensoid particles of euglobulin passing through the filter candles will impart an opalescent appearance to the finished product, and will gradually be deposited.

Also, I drew attention to the variability of the precipitating power of 30 and 31 % of saturation with ammonium sulphate. It was noticed that with sera in which there had been a comparatively slight removal of protein by 30 or 31 % of saturation with ammonium sulphate, the heated serum mixtures without exception filtered badly. The addition of 1½ to 2 % of sodium chloride to the serum mixtures before heating remedied this defect.

From these and from other observations it was concluded that the reaction of the sera and of the serum mixtures must be taken into account before the Banzhaf New One Fraction method could be depended on to furnish a permanently satisfactory end product. In this connection an investigation is being pursued as to whether the sodium chloride improves the filtration mainly by virtue of its specific action on globulins or by decreasing the hydrolysis of the phosphate of sodium to which the alkalinity is due. If the latter be the case then the desired improvement in filtration should be obtained by the addition to the medium of the amount of acid necessary to adjust the reaction to that required for optimum precipitation.

As the experimental work involved in this investigation must of necessity extend over a considerable period of time, I have, in the meantime, sought for some better method of removing from the serum mixture a relatively greater amount of protein without increasing the loss of antitoxin and without adding to the labour involved: the idea being to diminish as far as possible the amount of euglobulin in opalescent suspension in the final product.

The necessary clue was furnished by a study of the changes taking place in the solubility of serum proteins during the heating of serum or plasma (Table I). The changes may be summarised as follows:

(1) If serum or plasma be heated just to a temperature of 61° or 63° C. and kept at that temperature for a few minutes only, there is no appreciable change in the solubility of the serum proteins neither is there any change in the precipitating power of 30 % of saturation with ammonium sulphate. The heating process merely serves to completely aggregate the particles of precipitated euglobulin into a filterable form.

(2) If diluted serum or plasma be heated to a temperature of 61° or 63° C. and be kept at those temperatures for about 1 hour there is a considerable change, about 25 %, in the precipitating power of 30 % of saturation with ammonium sulphate. There is also a corresponding change if the serum or plasma be heated just to 67° (about 15 to 20 %).

(3) If serum or plasma diluted with one-third its volume of water be heated to a temperature of 55° C. for 15 hours there is only a slight change in the solubility of the serum proteins. But there is a 10 % increase in the precipitating power of 30 % saturation with ammonium sulphate.

(4) If diluted plasma or serum be heated to a temperature of 56°–57° for 15 hours, or to 58° for 7 to 8 hours, there is again only a slight change in the solubility of the serum proteins. But, the precipitating power of 30 % ammonium sulphate is increased from 30 to 49 %.

The changes in (4) are comparable with those observed by Banzhaf (1908). He regards them in the light of a conversion of pseudo- into euglobulin, but in view of the work of Hardy (1899), Chick and Martin (1912) it is more probable that the changes taking place in (1), (2), (3) and (4) are of the nature of a heat denaturation of proteins.

In devising a new method for the concentration of antitoxic sera there were, in view of my experimental observations, the following factors to be borne in mind:

(1) The complete aggregation under the influence of heat of the suspensoid particles of precipitated euglobulin into conglomerates sufficiently large to be retained on the surface of filter paper.

(2) The heat denaturation of serum proteins whereby their precipitability by 30 % saturation with ammonium sulphate is considerably increased.

(3) The stimulating effect of the addition of 1·5 to 2 % of solid sodium chloride on the process in (2)[1].

Experimental work was then undertaken in order to ascertain the most favourable conditions under which to ensure the denaturation of the proteins without appreciable loss of antitoxin. It was found that the more rapid denaturation induced by heating the serum or plasma at 61° or 63° C. for an hour was followed by an appreciable loss of antitoxin. With the prolonged heating of the serum or plasma at 55° C. there was a slight loss only of antitoxin but at the same time the denaturation of the proteins was only 10 %. At temperatures between 56°–58° C. there was, comparatively speaking, a slight loss only of antitoxic units while there was a considerable denaturation of protein.

The technique adopted.

The serum or plasma, diluted with one-third or even with only one-fifth its volume of water, and made 1·5 to 2 % with solid sodium chloride, is heated to a temperature of 56°–57° C. for 15 hours or to 57°–58° C. for 8 hours[2] (Stage 1).

During the prolonged heating a considerable change in the precipitability of the proteins occurs (Tables II *a* and *b*).

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[1] Our experiments so far are in favour of the adjustment of the medium by means of salt rather than by the addition of acid.

[2] We prefer to heat the serum or plasma at the lower temperature for the longer period. But, in the routine work it is sometimes more convenient to make arrangements for an 8 hours' than for a 15 hours' heating.

It is not really necessary to dilute the plasma or serum in order to ensure the required change during the prolonged heating, for the heat denaturation takes place to the same extent in the undiluted as in the diluted fluid. But in order to obtain the best conditions

The heated plasma is then made 30 % of saturation with ammonium sulphate and the mixture is heated to a temperature of 61° C. and kept at that temperature for a few minutes only (Stage II).

By raising the temperature of the serum-ammonium-sulphate mixtures to 61° the aggregation of the particles of suspensoid protein into a precipitate which can be readily separated by filtration through paper takes place without the precipitation of a further amount of soluble protein and without loss of antitoxin.

The mixture after cooling to 40°–45° C. is filtered. The precipitate is washed with 33 % of saturation with ammonium sulphate. The washings, after being filtered, are added to the main bulk of the filtrate which is then made 50 % of saturation with ammonium sulphate.

The resulting precipitate is filtered off, pressed and dialysed. The pressed precipitate has a yellowish colour and not the bluish green colour of Banzhaf's product.

To the residues from dialysis are added the necessary amounts of salt and preservative. The final products in bulk are of a reddish brown colour and do not exhibit even a trace of an opalescent suspension. In layers of about 1 to 2 inches thick the liquids are transparent and are of a yellowish brown colour.

The filtration of these products through pulp and through Pasteur-Chamberland filters, up to the time of writing, has presented no difficulty. Moreover the dilution of the dialysates with two or three times their bulk of 1 % saline does not impair their ease of filtration. In this respect alone the final product is superior to that obtained in the Banzhaf One Fraction process.

In the method thus advocated in this paper there have been incorporated the important factors in the Banzhaf-Gibson Two Fraction method, the Banzhaf One Fraction method and in my modification of the latter.

The method was carried out at first on an experimental scale and promised success: it was then used with a batch of 10 litres of oxalated plasma. It was found that, during the process the removal of about

for a satisfactory precipitation and subsequent heat aggregation of the protein precipitated by 30 % saturation with ammonium sulphate it is, in our opinion, necessary to make the above dilution with water before adding the necessary amount of ammonium sulphate solution.

So far as the denaturation without loss of antitoxin is concerned the same results may be obtained by making the plasma or serum 30 % of saturation with ammonium sulphate previous to Stage I of the heating process. But in this case the end product is not so satisfactory: it is distinctly opalescent.

70 % of the total proteins of the original plasma was effected, and at the same time, even though the precipitate from the 30 % saturation with ammonium sulphate had not been extracted, there was only a loss of 10 % of antitoxic units. The end product was so satisfactory that routine concentrations were carried out by this method.

Several batches of plasma and of serum, each of 100 litres, have been concentrated so far with excellent results both for tetanus and for diphtheria antitoxic sera. The details of typical concentrations carried out by this method and by the Banzhaf One Fraction process are given in Table III.

The denaturation of the serum proteins during the prolonged heating of the serum and their consequent increased precipitation by 30 % of saturation with ammonium sulphate has led to the preparation of a final product in which the protein content is about 17–19 % while the unitage per c.c. has been increased to 8–9 times that of the original serum. Moreover if the process be carried out with the customary precautions against undue loss and if all precipitates, filter papers, etc., used in the process be carefully washed with 33 % ammonium sulphate and added to the main filtrates from the first precipitate, the loss of antitoxic units need not be greater than 10 %; in fact it can be reduced to as little as 5 %.

Furthermore, the final product is a clear limpid fluid which, even on dilution with 2 or 3 parts of a 1 % salt solution, shows no trace of opalescence and therefore should remain permanently clear.

The method given above is an improvement on

A. *The Gibson-Banzhaf Method for four reasons.*

(1) There is only one fraction to consider and the extraction of the first precipitate with brine is no longer necessary. This involves the saving of time, labour and materials.

(2) The heating of the serum made 30 % of saturation with ammonium sulphate serves to agglutinate the suspensoid particles of protein into conglomerates sufficiently large to be retained by filter paper. In the Gibson-Banzhaf process, in order to ensure the retention of these particles, it was often necessary to increase the percentage of ammonium sulphate to 35.

(3) The potency of the finished product is nearly *twice* as great as that obtained by the Banzhaf-Gibson process while the protein content is only 17 to 19 %.

(4) The total loss of antitoxic units need not be greater than 10 %.

And on

B. *The Banzhaf One Fraction Process for three reasons.*

(1) By the removal of a much greater amount of protein, as a result of the heat denaturation of serum proteins and their consequent increased precipitability by 30 % of saturation with ammonium sulphate, there is less likelihood of filtration difficulties.

(2) By the addition of $1\frac{1}{2}$ to 2 % of sodium chloride to the serum mixture there is an adjustment of the reaction of the medium towards the conditions required for more complete precipitation of the particular proteins thrown out of emulsoid solution by the 30 % saturation with ammonium sulphate. This factor has a beneficial effect on filtration.

(3) The finished product is nearly *twice* as potent per c.c. as that obtained by the concentration of the same sera by Banzhaf's One Fraction method without containing a higher percentage of protein.

SUMMARY.

The method for the concentration of antitoxic sera suggested in this communication and now being used in this laboratory, presents a further step towards the desired goal, viz. the preparation, on a commercial scale for general therapeutic use, of antitoxic sera with a minimal amount of attendant protein.

In the concentration of sera on a large scale by the routine methods hitherto published the final products have shown a protein content of about 18 to 20 % with a potency per c.c. four to five times that of the original serum. The above described method yields, as a matter of routine, a final product with a protein content of about 17 to 19 % and with a potency per c.c. as much as nine times that of the original serum. With the use of better processes the potency might be higher still.

It is worth while to concentrate by this method plasma or sera in which the unitage is so low that hitherto they would have been discarded as having too low a potency for use even after concentration.

The patient will receive a relatively higher percentage of antitoxic units per gram of protein than if the sera had been concentrated by other methods, for by this method the removal of 70 % of the total proteins is ensured.

The heat denaturation of the proteins during the prolonged heating of the serum and consequent diminution of protein relatively to antitoxin may serve to minimise troubles with serum sickness.

In conclusion I desire to express my thanks to Dr A. T. MacConkey for the interest he has taken in the work and for the facilities accorded to me for the furtherance of the investigation.

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TABLE I.

The effect of the heating of serum or oxalated plasma (a) *on the solubility of the serum proteins and* (b) *on their precipitability by* 30 % *of saturation with ammonium sulphate.*

					(*a*)		(*b*)	
	Percentage protein content of serum or plasma	Proportion of water added to the serum or plasma before heating	Temperature to which diluted serum or plasma was heated	Duration of the time of heating at the chosen temperature	Percentage of protein in solution, calculated from the Refractometer Readings and expressed in terms of the original serum or plasma	Percentage conversion of soluble into insoluble protein during the heating process	Percentage of protein in solution in the filtrates from the serum or plasma in (*a*) made 30 % of saturation with ammonium sulphate. Calculated from the Refractometer Readings and expressed in terms of the original plasma or serum	Percentage increase in the precipitation of protein by 30 % of saturation with ammonium sulphate
I	7·78	$\frac{1}{2}$	Room temperature		7·78	—	6·11	
	7·78	$\frac{1}{2}$	63° C.	5 minutes	7·52	3·3 %	5·92	3·0 %
II	6·49	$\frac{1}{2}$	Room temperature		—	—	5·76	
	6·49	$\frac{1}{2}$	63° C.	$\frac{3}{4}$ hr	—	—	4·28	24·0 %
III	6·23	$\frac{1}{2}$	Room temperature		—	—	4·98	
	6·23	$\frac{1}{2}$	67° C.	5 minutes	—	—	4·00	19·8 %
IV	7·39*	$\frac{1}{5}$	Room temperature		7·39	—	5·35	
	7·39	$\frac{1}{5}$	55° C.	15 hrs	6·89	6·7 %	4·81	10·0 %
V	7·38	$\frac{1}{3}$	Room temperature		7·38	—	5·12	
	7·38	$\frac{1}{3}$	56°–57° C.	4 hrs	7·05	4·5 %	—	
	7·38	$\frac{1}{3}$	56°–57° C.	8 hrs	7·01	5·0 %	4·82	5·5 %
VI	6·13	$\frac{1}{3}$	Room temperature		6·13	—	5·78	
	6·13	$\frac{1}{3}$	56°–57° C.	6 hrs	5·99	2·3 %	5·48	5·2 %
	6·13	$\frac{1}{3}$	56°–57° C.	15 hrs	5·79	5·5 %	3·52	39·1 %
VII	7·10	$\frac{1}{3}$	Room temperature		7·10	—	5·52	
	7·10	$\frac{1}{3}$	58° C.	7–8 hrs	6·99	1·5 %	3·76	31·9 %
VIII	7·59*	$\frac{1}{5}$	Room temperature		—	—	4·88	
	7·59	$\frac{1}{5}$	58° C.	7–8 hrs	—	—	3·56	28·7 %

* In these cases an addition of 1·5 % of sodium chloride had been made.

TABLES II *a* AND *b*.

Changes in the serum proteins during the two stages of the heating process adopted in the method described in this paper.

In Table II *a*, during Stage I, the diluted oxalated plasma or serum, to which had been added 2 % of salt, was heated to 56°–57° for 15 hours. In Table II *b* this preliminary heating was conducted at a temperature of 58° for 7–8 hours.

In both cases the heated liquids were made 30 % of saturation with ammonium sulphate and the mixtures were then heated just to a temperature of 61° (Stage II).

TABLE II *a*. *Batch T.R.* 35.

	(i) Changes in the solubility of the Serum Proteins. Percentage of protein in solution calculated from the Refractometer Readings and expressed in terms of the original plasma	(ii) Changes in the precipitability of proteins by 30 % of saturation with Am_2SO_4. Percentage of protein in solution in the filtrates from making the serum in (i) 30 % of saturation with Am_2SO_4
STAGE I. *The prolonged heating of the diluted serum + 2 % NaCl at 56°–57° for 15 hours.*		
Serum + ⅓ its vol. of H_2O + 2 % NaCl (= A)	6·16	5·90
Serum + ⅓ its vol. of H_2O + 2 % NaCl heated to 56°–57° C. for 15 hours (= B)	5·90	3·90
STAGE II. *The rapid heating of B made 30 % with Am_2SO_4 just to 61° C.*		
Mixture B made 30 % of saturation with Am_2SO_4	—	3·90
do. do. heated just to 61° C.	—	3·90

Thus during the 15 hours' heating in Stage I there has been an *increased precipitation of* 34·5 % of the soluble protein by 30 % of saturation with ammonium sulphate. During the heating process in Stage II there has been no further conversion of soluble into insoluble protein.

TABLE II *b*. *Batch T.R.* 38.

	(i) Changes in the solubility of the Serum Proteins. Percentage of protein in solution calculated from the Refractometer Readings and expressed in terms of the original plasma	(ii) Changes in the precipitability of proteins by 30 % of saturation with Am_2SO_4. Percentage of protein in solution in the filtrates from making the serum in (i) 30 % of saturation with Am_2SO_4
STAGE I. *The prolonged heating of the diluted serum* + $1\frac{1}{2}$ % *NaCl at* 57°–58° *for* 8 *hours.*		
Serum + $\frac{1}{3}$ its vol. of H_2O + $1\frac{1}{2}$% NaCl (= A)	7·10	5·52
Serum + $\frac{1}{3}$ its vol. of H_2O + $1\frac{1}{2}$% NaCl after being heated to 57°–58° C. for 8 hours (= B)	6·99	3·76
STAGE II. *The rapid heating of B made* 30 % *with* Am_2SO_4 *just to* 61° *C.*		
B made 30 % of saturation with Am_2SO_4	—	3·76
do. do. and heated just to 61° C.	—	3·76

During the 8 hours' heating of the serum in Stage I there has been *an increased precipitation of* 31·3 % of the soluble protein by 30 % of saturation with ammonium sulphate. During the heating process in Stage II there has been no further conversion of soluble into insoluble protein.

TABLE III.

A comparison between the results obtained for the concentration of antitoxic sera by the Banzhaf One Fraction method and by the method advocated in this paper.

Sera concentrated by Banzhaf's One Fraction method	**Sera concentrated by the new method given above**
A. *Diphtheria Antitoxic Sera.*	
Batch No. D.R. 31 (50 litres of oxalated plasma)	Batch No D.R. 33 (100 litres oxalated plasma)
Before concentration :	*Before concentration :*
Unitage per c.c. = 500	Unitage per c.c. = 275
Protein content = 7·00 %	Protein content = 7·39 %
After concentration :	*After concentration :*
Unitage per c.c. = 2000	Unitage per c.c. = 2200
Protein content = 20·10 %	Protein content = 17·5 %
Loss of antitoxic units during the process = 12 %	**Loss of antitoxic units during the process = 8 %**
Amount of protein removed = 25·4 % of total	**Amount of protein removed = 70·4 % of total**
Potency increased by 4 times	**Potency increased by 8 times**
B. *Tetanus Antitoxic Sera.*	
Batch No. T.R. 31 (100 litres)	Batch No. 35 T.R. (100 litres)
Before concentration :	*Before concentration :*
Unitage per c.c. = 175	Unitage per c.c. = 100
Protein content = 6·99 %	Protein content = 6·47 %
After concentration :	*After concentration :*
Unitage per c.c. = 850	Unitage per c.c. = 900
Protein content = 20·2 %	Protein content = 19·0 %
Loss of antitoxic units during the process = 20 %*	**Loss of antitoxic units during the process = 10 %**
Amount of protein removed = 40·4 % of total	**Amount of protein removed = 68·9 % of total**
Potency increased by 5 times (nearly)	**Potency increased by 9 times**

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* In this particular concentration the percentage loss of antitoxic units was greater than usual. Our average loss by the Banzhaf One Fraction process is of the order of 10 %.

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No 7

ON FACTORS LIMITING THE EXTENT OF THE CONCENTRATION OF ANTITOXIC SERA BY THE FRACTIONAL PRECIPITATION METHODS AT PRESENT EMPLOYED.

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The use of concentrated antitoxic sera in the treatment of diphtheria and tetanus has been generally recommended with a view to minimising the troubles of serum sickness. For, during the process of concentration of sera, there is a removal of the proteins not associated with antitoxin. It is thus possible to administer large doses of antitoxin with far less discomfort to the patient and with the introduction of a considerably smaller amount of the proteins of horse serum than if the original unconcentrated sera had been used.

The aim of any concentration process is therefore the preparation, on a commercial scale for therapeutic uses, of antitoxic sera which shall contain a minimum amount of attendant protein.

In a previous communication (Homer, 1916) it has been shown that, in the method recently adopted in this laboratory for the concentration of sera, a removal of from 65 to 70 % of the total proteins of the original serum is effected with a consequent increased potency eight to nine times that of the original serum.

The new method thus presented a step towards the desired goal, for by previous methods there had been a removal of about 40 % of the total proteins with a consequent increased potency of from four to five times. From experimental observations it was evident that the heat denaturation[1] of the serum proteins was the primary factor influencing the removal of protein beyond that obtained by the methods previously in vogue.

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[1] Throughout this paper the term "heat denaturation" is used as a convenient term for the increased precipitability of the serum proteins by 30 % of saturation with ammonium sulphate induced by the prolonged heating of serum.

Some definite relationship thus exists between the extent of the removal of unnecessary protein from the serum and the degree of concentration of antitoxin in the final product. Therefore the question arose as to whether the heat denaturation of the proteins could not be induced to an extent greater than that obtained in our new process so as to yield a final product in which the percentage of protein had been still further reduced.

In the course of experimental work I have ascertained that the heat denaturation of the serum proteins is considerably influenced by the presence of acid and of electrolytes. But, as was anticipated, the presence of acid during the prolonged heating of sera, even at 57–58° C., under some conditions has a deleterious effect on the antitoxin: a loss of as much as 50 % of the antitoxin has been measured. On the other hand, as is shown in Table I, in the presence of the electrolytes employed there was no appreciable destruction of antitoxin at a temperature of 57–58° C. Heating the serum in presence of 30 % of ammonium sulphate to a temperature of 61° and 63° for 2 hours resulted in the destruction of 20 % of the antitoxin. With 10 to 20 % of sodium chloride there was no loss after 2½ hours' heating at 62° C. But, when the serum was saturated with salt and heated to 62° for 2½ hours the mixture coagulated; the residue after dilution with an equal bulk of 1 % saline, was tested for its antitoxin value and showed a loss of about 50 %.

In view of these results the use of sodium chloride as the denaturating agent was more carefully investigated.

Serum or plasma was diluted with one-third its volume of water, definite amounts of the electrolyte were added and the diluted serum was then heated to a temperature of 57–58° C. for periods of 7 to 8 hours. The heated liquid was made 30 % of saturation with ammonium sulphate, the temperature raised just to 60° C., and the mixture cooled to 50° C. and filtered. The precipitate (*First Fraction Precipitate*) was washed with 30 % saturated ammonium sulphate and the washings added to the main bulk of the filtrate. The filtrate and washings were then brought up to 50 % of saturation with ammonium sulphate and filtered. The precipitate (*Second Fraction Precipitate*) was pressed and dialysed. The residue from the dialysis of this precipitate was made 0·35 % with cresylic acid and the necessary amount of salt was added (*Final Product*).

The protein and the antitoxic contents of the final products were estimated in the usual way and were compared with those of the original serum.

TABLE I.

The loss of antitoxic units during the prolonged heating of antitoxic sera to which electrolytes have been added.

In each case the serum or plasma was diluted with one-third its volume of water previous to the addition of the electrolyte.

	Electrolytes added to the diluted plasma	Temperature to which the plasma, after the addition of the electrolyte, was heated	Duration of the time of heating at the specified temperature	Unitage of the original serum	Percentage loss of antitoxic units during the heating process
Tetanus:					
Adelaide 1/6/16	1½ % NaCl	57–58° C.	8 hours	150	negligible
Adelaide 1/6/16	4 ,,	,,	8 ,,	150	,,
Diphtheria:					
△ R. 41	2 ,,	,,	8 ,,	250	,,
△ R. 42	5 ,,	,,	4 ,,	250	,,
△ R. 40	2 ,,	,,	8 ,,	500	,,
Lille 26/6/16	8 ,,	,,	8 ,,	150	less than 10 %*
Lille 26/6/16	20 ,,	,,	8 ,,	150	about 10 %
Ethel 2/5/16	20 ,,	,,	8 ,,	350	negligible
Lille 26/6/16	Saturated with NaCl	,,	8 ,,	150	,,
Ethel 2/5/16	Saturated with NaCl	,,	8 ,,	350	,,
Lille 26/6/16	2 % NaCl + 30 % AM_2SO_4	,,	8 ,,	150	,,
Ethel 2/5/16	2 % NaCl + 30 % AM_2SO_4	61°	2 ,,	350	20 %
Ethel 2/5/16	2 % NaCl + 30 % AM_2SO_4	63°	2 ,,	350	,,
Ethel 10/10/16	10 % NaCl	62°	2½ ,,	300	negligible
Ethel 10/10/16	20 % NaCl	,,	2½ ,,	300	,,
Ethel 10/10/16	Sat. NaCl	,,	2½ ,,	300	about 50 %

* Not tested out.

TABLE II *a*.

The precipitation of serum proteins by the addition of sodium chloride to plasma (1) *previously diluted with half its volume of water, and* (2) *previously diluted with half its volume of water and made* 30 % *of saturation with ammonium sulphate.*

Percentage of NaCl added to the diluted plasma.	(1) Percentage of Residual Protein in solution in the plasma after the addition of the various percentages of NaCl. Expressed in terms of the original plasma	(2) Percentage of Residual Protein in solution after making the salted plasma 30 % saturation with ammonium sulphate. Expressed in terms of the original plasma
0	7·70	6·20
1	7·68	6·20
2	7·68	5·50
3	7·65	5·38
4	7·37	5·37
5	7·32	5·32
6	7·32	5·30
7	7·32	4·15
8	7·19	3·75
9	6·99	3·70
10	6·86	3·70

TABLE II *b*.

Showing the influence exercised by sodium chloride on the precipitation of antitoxin along with the First Fraction Precipitate *both* (1) *before and* (2) *after the prolonged heating of the serum.*

Serum	Unitage	Percentage of salt added to the diluted plasma	Unitage of the filtrate from the First Fraction Precipitate
(1) *The unheated plasma:*			
Adelaide 25/5/16 Tetanus	150	4	150 (guinea pig used in testing the serum for this unitage died in 90 hours)
Adelaide 25/5/16 ,,	150	7	150 (guinea pig used in testing the serum for this unitage died within 48 hours)
(2) *The heated plasma:*			
Adelaide 1/6/16 Tetanus	150	2	145 nearly (guinea pig used in testing the serum for this unitage died in 90 hours)
Adelaide 1/6/16 ,,	150	4	145 just
Adelaide 1/6/16 ,,	150	8	100 (guinea pig used in testing the serum for this unitage died within 90 hours)

Where the percentage of sodium chloride added to the serum had been greater than 5 it was found advisable to dilute the heated liquid before the addition of ammonium sulphate for the first precipitation. Otherwise as is shown in Tables II*a* and II*b* there is an increased precipitation of protein and of antitoxin in the first precipitate both in the unheated and in the heated plasma.

In Table III*a* have been incorporated the results of those concentrations in which the antitoxic units of the original serum were, within the limits of experimental error, fully accounted for in the final product.

In Table III*b* have been embodied the results of concentrations in which, although there had been no loss of antitoxin during the heating process, the final product showed an appreciable loss of antitoxic units.

A further investigation, however, demonstrated that in the concentrations thus studied the missing antitoxin had been brought down in the First Fraction Precipitate. None was detected in the albumin fraction (filtate from Second Fraction Precipitate). Moreover, the antitoxin thus brought down with the First Fraction Precipitate could not be recovered by washing the precipitate with 30 % saturated ammonium sulphate: for this purpose extraction with saturated salt solution was essential.

A study of the data in Tables III*a* and III*b* reveals the fact that *there is a limit to the extent to which the removal of the serum proteins can be effected without disturbing the precipitation of the antitoxin with the Second Fraction Precipitate.* For, it is apparent that if the percentage removal of the total serum proteins be increased much beyond 80 %, there is a consequent increased precipitation of antitoxin in the First Fraction Precipitate.

It is also obvious from Table III*a* that the order of the increased potency measured in the Final Product depends upon two factors. The one factor is the extent of the denaturation induced during the heating process and its effect on the removal of proteins during the concentration processes. The other factor is the efficacy of the pressing of the Second Fraction Precipitate before dialysis.

From the results thus recorded it will be seen that it has been possible to concentrate sera by as much as 12 times while the percentage protein content of the Final Product has not been greater than 20.

In an experimental concentration in which it was calculated that the potency would be increased by 15 times it was however found to be only 8½ times that of the original. The antitoxin, thus not present

TABLE III *a*.

Showing the percentage of the total antitoxic units of the original serum recovered in the Second Fraction Precipitate *in cases where the percentage removal of the proteins of the original serum during the concentration process has not been greater than* 80.

	Unitage of the original serum	Percentage protein content of the original serum	Percentage of electrolytes added to the plasma	Percentage loss of antitoxic units during the heating process	Extent of the heat denaturation	Percentage removal of the total serum proteins during the concentration	*Second Fraction Precipitate after Dialysis*			Percentage of the total original antitoxic units recovered in the Second Fraction Precipitate
							Percentage protein content	Unitage per c.c.	Increased potency per c.c. over that of the original serum	
Tetanus:										
Adelaide	150	6·16	8 % NaCl	negligible	48 %	79·0	13·3	1800	12 times (nearly)	95
Diphtheria:										
Lille 26/6/16	150	7·26	8 % ,,	,,	35 %	80·0	14·24	1100	7⅓ ,,	76
Lille 26/6/16	150	7·26	12 % ,,	,,	40 %	71·0	17·3	1600	10½ ,,	94
Lille 26/6/16	150	7·26	20 % ,,	about 10 %	not estimated	70·0	15·0	900	6 ,,	80
Smyrna* 7/7/16	600	9·68	7 % ,,	not estimated	2 %	68·2	20·0	2750	4½ ,,	75
Lille 26/6/16	150	7·26	30 % Am_2SO_4 2 % NaCl	negligible	20·6 %	71·0	10·5	800	5·6	87·5
Tetanus:										
T.R. 35	100	6·61	2 % NaCl	not tested†	42·2 %	68·9	19·5	900	9 ,,	90
T.R. 36	150	7·59	,,	,, †	28·7 %	70·0	19·2	1300	8½ ,,	80
T.R. 37	200	7·10	,,	,, †	31·3 %	60·7	18·04	1300	6½ ,,	91
T.R. 38	100	6·60	,,	,, †	32·0 %	72·0	15·62	850	8½ ,,	85
Diphtheria:										
△ R. 33	275	—	,,	,, †	—	70·4	17·5	2200	8 ,,	92
△ R. 39	400	8·51	,,	,, †	31·1 %	77·3	19·33	3000	7½ ,,	80
△ R. 40	500	7·57	,,	negligible	23·5 %	60·0	17·0	2500	5 ,,	85
△ R. 42*b*	250	6·60	5 % NaCl	negligible	41·2 %	70·0	18·6	2250	8½ ,,	86

* Concentrated by Banzhaf's 1913 Method.

† We have so often satisfied ourselves that there is no appreciable loss of antitoxin during the heating of serum to which has been added 2 % of NaCl, to a temperature of 57–58° C. for a period of 7–8 hours, that we consider it as established that at this stage in the concentration there is no loss of antitoxin.

TABLE III *b*.

Showing the percentage of the total antitoxic units of the original serum recovered in the Second Fraction Precipitate *in cases where the percentage removal of the proteins of the original serum during the concentration process has been greater than* 80.

	Unitage of the original serum	Percentage protein content of the original serum	Percentage of NaCl added to the diluted plasma	Temperature to which the plasma was heated	Duration of the heating at the specified temperature	Percentage loss of antitoxic units during the heating process	Extent of the heat denaturation	Percentage of the total proteins removed during the process of concentration	Percentage of original antitoxin units appearing in the dialysed Second Fraction Precipitate	Presence or absence of antitoxin in the First Fraction Precipitate which requires to be extracted with brine
Diphtheria:										
Lille 26/6/16	150	7·26	20	57–58° C.	8 hours	about 10 %	51 %	83·2	60	+ ve*
Ethel 2/5/16	350	8·62	20	,,	,,	negligible	50 %	90†	23	+ ve (found to be 53·5 %)
Lille 26/6/16	150	7·26	saturated with NaCl	,,	,,	negligible	60 %	83·6	50	+ ve*
Ethel 2/5/16	350	8·62	saturated with NaCl	,,	,,	negligible	not tested	86·8	44	+ ve (at least 20 %)*
Δ R. 35	300	6·60	10 % NaCl and 0·3% Trikresol	,,		negligible	50 %	89·5	66	+ ve*
Tetanus:										
Adelaide 1/6/16	150	6·16	8 %	,,	,,	negligible	50 %	85·1	70	+ ve*

* Not tested out because of scarcity of experimental guinea-pigs.

† The heated serum was not diluted before precipitating with ammonium sulphate.

in the final product, was found to be in the First Fraction Precipitate. The phenomenon was somewhat unexpected as the percentage removal of total serum proteins was not greater than 66 and was well within the limit indicated in Table III*a*.

This observation, in conjunction with those given in Table III*b*, naturally suggested that there might be a maximum load of antitoxin which could be carried by a fixed amount of protein in the Final Product obtained by the Fractional Precipitation Methods at present in use for the concentration of sera.

In order to elucidate this point experimental concentrations of high potency antidiphtheritic sera were carried out. The sera were concentrated by Banzhaf's One Fraction Method which will yield a concentration of about 4–5 times with a removal of about 40–50 % of the total serum proteins. The same sera were also concentrated by my method which, with a heat denaturation of 30 to 40 % will yield a concentration of about 8 times.

From the results of these concentrations, which have been embodied in Table IV, it appears that *there is a limit to the amount of antitoxin which can be associated with a given weight of protein in the Final Product*: this maximum amount is of the order of 20,000 to 25,000 diphtheria antitoxin units per gramme of protein.

The possible load of tetanus antitoxin units per gramme of protein beyond 13,000 units has not been worked out as high potency tetanus antitoxic sera could not be spared at the present time for experimental work.

In those cases, Table IV, in which the load, by estimation, should have been of the order of 30,000 units per gramme of protein or more, it was found that the excess of units beyond the aforesaid limit had been transferred to the First Fraction Precipitate in spite of the fact that the percentage removal of proteins was less than 80.

We thus see that, by the fractional precipitation methods at present in vogue for the concentration of antitoxic sera, the degree of concentration is limited by two factors:

(1) The extent of the removal of the total proteins of the original serum; and

(2) the maximum load of antitoxic units which can be associated with one gramme of protein in the final product.

There will be transference of antitoxin to the First Fraction Precipitate if the percentage removal of proteins be greater than 80 or if

TABLE IV.

The maximum load of antitoxin which can be associated with one gramme of protein in the residues from the dialysis of the Second Fraction Precipitate (*i.e. in the* Final Product), *in the concentration of antitoxic sera by the Fractional Precipitation Methods now in vogue.*

	Unitage of the original serum per c.c.	Protein content of the original serum	Concentration process adopted	Denaturation induced during the heating process	Percentage of the total protein of the original serum removed during the concentration process	*Anticipated* unitage of the final product per c.c	In the Final Product was found			Percentage of the total antitoxic units of the original serum appearing in the Final Product	Antitoxin carried down with the First Fraction Precipitate not extractable with 30 % of ammonium sulphate but extractable with brine
							Unitage per c.c	Protein content	Number of antitoxic units per gramme of protein		
		%		%				%			
Aurora 19/6/16	1100	10·03	Banzhaf (1913)	1·0	47	3500	3500	17·6	20,000	93	negligibl
Aurora ,,	1100	10·03	†Homer (1916)	13·0	54·5	4500	4250	19·8	21.500	93	..
Aurora ,,	1100	10·03	†Homer (1916)	44·0	70	6600	4750	20·2	23,500	65	22·5 %
Aurora 21/8/16	900	8·18	Banzhaf (1913)	2·0	40	3600	3000	17·2	18.300	85·5	negligibl
Aurora ,,	900	8·18	†Homer (1916)	34·5	56	5400	4750	21·3	22,300	88	..
Lloyd George 5/9/16	800	8·74	Homer (1916)	37·0	72	7000	4400	20·8	21.200	59	+ ve*
Aurora 5/9/16	700	7·08	†Homer (1916)	30·0	70	6000	3600	20·6	17.500	57	+ ve*
San 5/9/16	750	7·26	Homer (1916)	41·0	70	6500	4500	21·1	21.300	66·5	− ve*

* Owing to scarcity of experimental guinea-pigs these products were not tested out

† We have many times demonstrated that there is no appreciable loss of antitoxin during the prolonged heating of sera by my method. This was again verified with the concentrations of the sera thus indicated.

the load of antitoxin threatens to be greater than of the order of 20,000 to 25,000 units per gramme of protein[1].

In a previous communication (Homer, 1916) I have drawn attention to the necessity for a study of the hydrogen ion concentration of the sera to be concentrated. Additional evidence in support of this point has been forthcoming during the progress of the present investigation. It will be seen (Table III *a*) that, in routine concentrations, the heat denaturations varied from 20 to 45 % and as a matter of practical experience it has been found that the most satisfactory end products, from the standpoint of filtration, were obtained where the heat denaturations had been of the order of 30 to 40 %.

I have ascertained that these variations are due to differences in hydrogen ion concentrations of the respective sera. Moreover, by altering the reaction of the sera it is possible to produce wide variations in the heat denaturations, although, apparently, the heat denaturation cannot be induced beyond certain limits without destruction of antitoxin.

The adjustment of the reaction of the batches of sera for concentration to a definite hydrogen ion concentration is proving of much value as it leads to uniform and consistent results. The details of the procedure adopted for the standardisation of the concentration process by an adjustment of the hydrogen ion concentration of the sera will form the subject of a further communication.

SUMMARY.

The following conclusions of practical value to those engaged in serum concentration can be drawn from the experimental work described above:

(1) An end product containing not more than 20 % of protein and having a potency of 8 or 10 times that of the original can be obtained as a matter of routine where the pooled antidiphtheritic sera has a unitage not greater than 500, and where the heat denaturation is of the order of 40 %.

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[1] Banzhaf and Gibson (1907) working on an experimental scale with small quantities of unheated serum, state that, by successively fractionally precipitating with ammonium sulphate within narrow limits (50 + and 50–56 % of saturation), they have obtained a high potency fraction of the order of 45,000 units per gramme of protein.

Up to the present, working with heated sera, I have been unable to obtain a fraction with so high a unitage per gramme of protein.

(2) By the fractional precipitation methods at present employed for the concentration of antitoxic sera for therapeutic use the degree of concentration of high potency sera cannot be taken beyond the limit of about 22,500 units of antitoxin per gramme of protein in the end product.

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V. THE REACTION OF SERA AS A FACTOR IN THE SUCCESSFUL CONCENTRATION OF ANTITOXIC SERA BY THE METHODS AT PRESENT IN USE.

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In a previous communication [1916, 1] I suggested that the practical difficulties so often encountered in the filtration of concentrated antitoxic sera might be due to the fact that hitherto no cognisance had been taken of the reaction of the sera before being concentrated.

With this object in view experiments have been carried out in which the effect of variations in the reactions of the sera have been considered with reference to:

(i) The precipitation of serum proteins by 30 per cent. of saturation with ammonium sulphate.

(ii) The increased precipitation of the serum proteins during the shortened heating process in the Banzhaf (1913) method. [Banzhaf, 1913.]

(iii) The increased precipitability of the serum proteins during the preliminary heating of the plasma as suggested in a previous paper. [Homer, 1916, 2.]

(iv) The practical application of the results obtained from the above investigations.

During the investigation of these points there has been accumulated a considerable amount of data which shows that the reaction of the serum is an important factor in the successful precipitation and heat denaturation of the serum proteins and that, unless the reaction be in some way controlled, apparently inexplicable irregularities are apt to occur in the course of the concentration.

1. THE EFFECT OF CHANGES IN THE REACTION OF SERUM ON THE PRECIPITATION OF THE SERUM PROTEINS BY 30 PER CENT. OF SATURATION WITH AMMONIUM SULPHATE.

Several series of experiments were conducted with oxalated plasma from different horses and from batches of pooled plasma or serum made up for concentration.

In each series, separate volumes of the test plasma were treated with known volumes of acid and of alkali. The plasma, thus treated, was brought up to 30 per cent. of saturation with ammonium sulphate by the addition of the necessary volume of a saturated solution of ammonium sulphate. The hydrogen ion concentration of the plasma and of the plasma-ammonium-sulphate mixtures was measured, wherever possible, by means of indicators and Sörensen's solutions in Walpole's colorimeter. The determinations, thus made, were subsequently checked by the electrical method as it was found that many of the indicators were rendered unreliable in the presence of so much protein as there is in serum. The protein contents of the filtrates from the serum mixtures were measured by means of the Zeiss immersion refractometer.

TABLE I.

Showing the influence of changes in the hydrogen ion concentration of the serum-ammonium-sulphate mixtures on the precipitation of serum proteins by 30 % *of saturation with ammonium sulphate.* (*Protein content of the plasma* = 7·92 %. *Hydrogen ion concentration of the saturated solution of ammonium sulphate added to the plasma* = p_H^+ 6·480.)

Volume of normal acid (or equivalent) added to 100 cc. of the diluted plasma	Volume of normal alkali (or equivalent) added to 100 cc. of the diluted plasma	p_H^+ of the diluted plasma to which the acid or alkali has been added, measured by the electrical method	p_H^+ of the filtrate from the serum-ammonium-sulphate mixtures, measured by the electrical method	Residual percentage of protein in solution in the filtrates from the serum-ammonium-sulphate mixtures, measured by means of the refractometer
—	19·6 cc. N . NH_3	—	8·754	5·75
—	13·1 ,,	—	8·551	5·80
—	6·55 ,,	9·945	8·455	5·90
—	3·28 ,,	9·789	8·022	6·00
—	1·96 ,,	9·304	7·745	6·00
—	0·98 ,,	8·500	7·400	6·30
0·00 cc. N . HAc	0·00 ,,	7·430	7·077	6·30
0·38 ,,	—	—	7·000	6·52
1·25 ,,	—	6·450	6·438	6·60
2·80 ,,	—	5·921	6·021	6·60
3·60 ,,	—	5·440	5·579	6·25
3·76 ,,	—	5·225	5·327	6·20
3·99 ,,	—	5·025	5·145	5·87
4·99 ,,	—	4·600	4·972	5·02
6·00 ,,	—	4·560	4·712	4·52

The data with respect to one of the series of experiments have been embodied in Table I; comparable results were obtained with all the samples of serum and plasma investigated.

A study of Table I shows that within certain limits (p_{H}^{+} 5·3 to 7·4) the reaction of the mixtures has little effect on the extent of the precipitation of the serum proteins. Beyond these limits in both directions there is an increased precipitation of protein by the sulphate. It was also noticed that the consistency of the precipitated euglobulin was greatly influenced by the reaction of the mixtures. The agglutination of the precipitate into a granular and readily filterable form proceeded most satisfactorily at a hydrogen ion concentration of about p_{H}^{+} 5·3 to 5·0, i.e. on the slightly acid side of the isoelectric point of euglobulin (p_{H}^{+} 5·5).

The state of aggregation of the Second Fraction precipitate (50 % of saturation with ammonium sulphate) was also dependent on the reaction of the mixture: the conditions most favourable for this precipitation are in the course of investigation.

II. THE EFFECT OF CHANGES IN THE REACTION OF THE SERUM ON THE INCREASED PRECIPITATION OF THE SERUM PROTEINS INDUCED DURING THE SHORTENED HEATING PROCESS IN BANZHAF'S (1913) METHOD.

Several series of experiments were undertaken with samples of oxalated plasma from individual horses and from batches of pooled plasma from different horses.

In each series, to separate volumes of the test plasma contained in stoppered bottles, was added a known amount of acid or of alkali together with the volume of a saturated solution of ammonium sulphate necessary to bring the sulphate content to 30 % of saturation. The hydrogen ion concentration and the protein content of the clear filtrates from each of the experimental liquids were measured in the usual way. Experiments were carried out both with undiluted plasma and with plasma diluted with one-third its volume of water.

The mixtures were heated and then filtered. The protein content of the filtrates was estimated and compared with the corresponding value for the unheated liquids. From the data thus obtained was calculated the percentage increased precipitation of the soluble proteins due to the heating of the mixtures.

Comparable results have been obtained with each of the sera examined irrespective of their protein content and of their dilution with water. The data with respect to one of the series of experiments have been embodied in Table II and represented graphically in the curve in Fig. 1.

TABLE II.

Showing the influence of changes in the reaction of the sera on the increased precipitation of protein during the heating of the serum-ammonium-sulphate mixtures to a temperature of 61° *and keeping them at that temperature for* 7 *to* 10 *minutes.* (*Protein content of the plasma* = 8·72 %. *The saturated solution of ammonium sulphate added to the plasma had a hydrogen ion concentration* p^+_{H} 6·480.)

Volume of normal acid (or equivalent) added to 100 cc. of plasma diluted with one-third its volume of water	Volume of normal acid (or equivalent) added to 100 cc. of the diluted plasma	p^+_{H} of the serum-ammonium-sulphate mixtures determined by the electrical method	Percentage increase in the precipitation of the soluble proteins induced during the heating of the serum-ammonium-sulphate mixtures	Remarks on the filtration of the heated serum-ammonium-sulphate mixtures
—	19·64 cc. N . NH_3	8·754	17·7	Readily, filtrate clear
—	13·04 ,,	8·577	16·4	,, ,, ,,
—	6·52 ,,	8·455	13·4	,, ,, ,,
—	3·28 ,,	8·022	10·0	,, ,, ,,
—	1·96 ,,	7·745	7·0	,, ,, very slightly opalescent
—	0·98 ,,	7·489	9·0	,, ,, ,, ,,
—	0·49 ,,	7·312	7·0	Less readily, filtrate slightly opalescent
0·00 cc. N . HAc	0·00 ,,	7·077	6·3	,, ,, ,, ,,
0·98 ,,	—	7·000	6·8	Slowly, filtrate opalescent
1·96 ,,	—	6·428	3·0	,, ,, ,,
2·94 ,,	—	6·021	2·8	,, ,, ,,
3·60 ,,	—	5·579	0·5	,, ,, ,,
3·76 ,,	—	5·327	18·0	Less slowly, filtrate slightly opalescent
3·92 ,,	—	5·145	20·7	Readily, filtrate clear
4·58 ,,	—	5·001	39·0	,, ,, ,,
4·88 ,,	—	4·972	52·6	,, ,, ,,
5·88 ,,	—	4·712	72·2	,, ,, ,,

It was noticed that where the hydrogen ion concentration of the mixtures was between the values p^+_{H} 5·3 and 7·0, the filtration of the heated liquids was slow and the filtrates were markedly opalescent. Throughout these ranges the conversion of soluble into insoluble protein was least. In those cases in which the reaction had been more acid or more alkaline than in the above the filtration of the mixtures was rapid and the filtrates were clear, while the conversion of soluble into insoluble protein was of an appreciable order. On the alkaline side between the ranges p^+_{H} 7·0 and 8·7 the conversion gradually rose from 5 to 17 %. On the acid side, as is seen at a glance in the curve in Fig. 1, the rate of increase was much more marked; it rose from 0·5 to 72 % between the ranges p_H^+ 5·5 and 4·7.

No trouble was experienced with the filtration of the final products obtained in the concentrations of sera in which the reaction of the serum mixtures previous to heating had been adjusted to p_H^+ 5·2 or p_H^+ 5·0. As was however to be anticipated from the character of the filtration of the hot serum mixtures, difficulties were encountered with the end products where the reaction of the serum mixtures had been between the limits p_H^+ 5·3 and 7·0. In these cases the unsatisfactory filtration of the serum mixtures may be ascribed to one of two possible causes; either that between these limits the hydrogen ion concentration is unfavourable for the complete agglutination of the precipitated proteins: or that, since near the isoelectric point the heat denaturation of the serum proteins is least, it is possible that in this region the proteins which, under more acid or more alkaline conditions, would be appreciably denaturated are in a transitory, semi-emulsoid, semi-rigid stage and as such impart a slimy consistency not only to the serum mixtures but also to the final products.

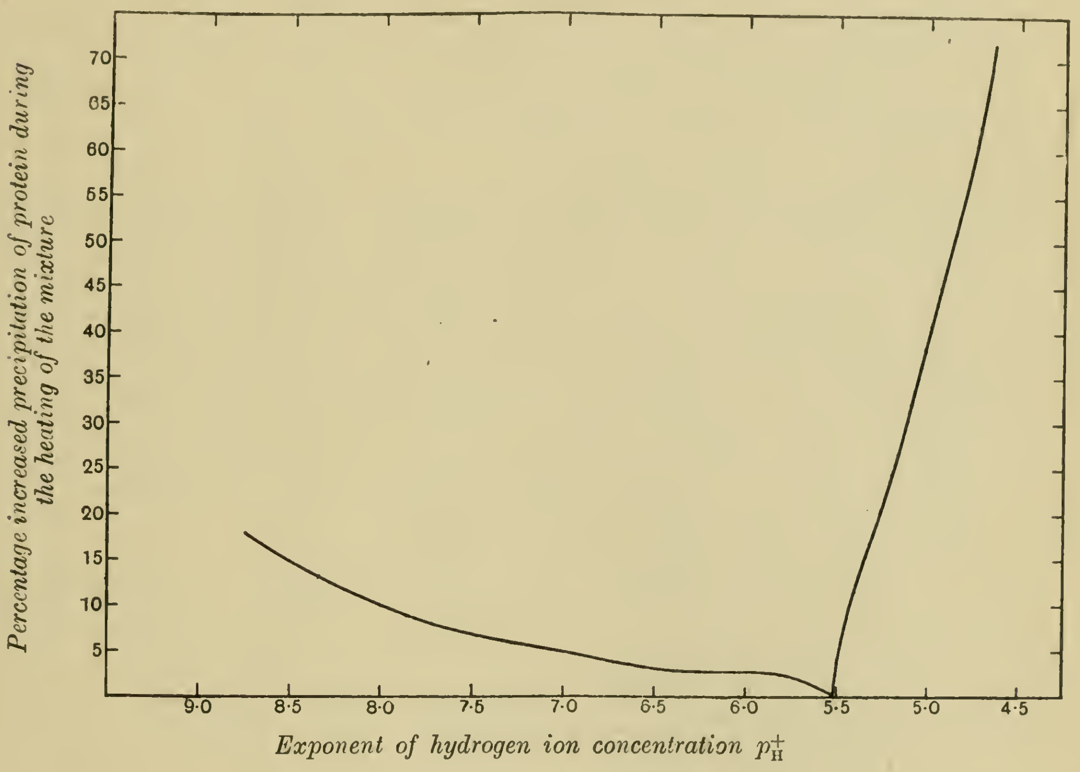

Fig. 1. Curve representing the influence of the reaction of the serum mixtures on the percentage increased precipitation of soluble proteins during the heating of serum-ammonium-sulphate mixtures by Banzhaf's method.

It was also found that, with serum mixtures more alkaline than p_{H}^{+} 8·0, there was difficulty with the filtration of the final product even though the filtrates from the heated serum mixtures were clear. In these cases, when these clear alkaline filtrates were acidified to a point p_{H}^{+} 5·5 an opalescence was produced. The presence of a certain amount of precipitated protein which had been kept in the disperse phase by the alkali was thus indicated. The colloidal aggregates thus dispersed escaped precipitation with the First Fraction precipitate but were subsequently precipitated with the Second Fraction precipitate. They impart to the residue from the dialysis of the latter a slimy consistency and thereby considerably impede filtration through filter candles.

III. THE EFFECT OF CHANGES IN THE REACTION OF SERA ON THE INCREASED PRECIPITABILITY OF THE SERUM PROTEINS INDUCED DURING THE HEATING OF SERUM TO A TEMPERATURE OF 57° FOR 6 HOURS.

During the course of our practical experience with the method suggested by me [Homer, 1916, 2] for the concentration of antitoxic sera it was observed that the heat denaturation of the serum proteins induced during the preliminary heating of the sera varied from 13 to 45 %. Throughout the winter and spring months the values with plasma varied from 28 to 35 % while with carbolised sera values as high as 45 % were reached. During the summer months when the horses were being given a considerable amount of green grass, the denaturation varied from 13 to 20 %.

After a considerable amount of experimental work it was ascertained that these variations were due to differences in the hydrogen ion concentration of the different plasmas and sera. During the summer months, when the low values for the heat denaturation were obtained, the oxalated plasma was slightly less alkaline than that obtained during the winter and spring months.

Samples of plasma from individual horses and from batches of the pooled plasmas from different horses were respectively diluted with one-third their volume of water. The hydrogen ion concentration of the diluted plasma was measured in each case. The reaction of the various samples was adjusted to the value of about p_{H}^{+} 5·6 to 5·9 and also to the value p_{H}^{+} 8·5. The original samples and the adjusted samples were heated in stoppered bottles to a temperature of 57° for 6 hours. The percentage increased precipitability of the serum proteins by 30 % of ammonium sulphate was measured with the aid of the Zeiss refractometer.

The data with respect to these observations have been embodied in Table III. They show that the variations in the extent of the heat denaturation of the serum proteins of individual sera can be obviated by adjusting the reaction of the sera to some fixed value. Thus, when the reaction was adjusted to about p_{H}^{+} 5·6 to 5·9 the denaturation was of the order of 10 %, while at the value p_{H}^{+} 8·5 it was of the order of 40 %[1].

TABLE III.

Showing that the variation in the heat denaturation induced during the heating of different samples of plasma at 57° for 6 hours can be overcome by an adjustment of the reaction of the sera to a fixed degree of acidity or of alkalinity.

			Percentage increased precipitability of the serum proteins during the heating of		
Sample No.	Percentage protein content of the plasma	Value for p_{H}^{+} of the diluted plasma	undiluted plasma	diluted plasma brought, by the addition of ammonia, to the hydrogen ion concentration p_{H}^{+} 8·5*	diluted plasma brought, by the addition of acetic acid, to the hydrogen ion concentration p_{H}^{+} 5·9*
1	8·59	7·648	31·1	—	—
2	8·12	7·503	28·9	44·0	13·0
3	7·80	7·460	26·4	41·0	9·0
4	8·59	7·563	28·4	40·0	12·0
5	6·56	7·430	22·3	41·0	13·0
6	7·57	7·346	23·5	40·5	—
7	7·92	7·346	22·0	—	8·7
8	6·55	7·863	39·0	43·0	13·0
9	6·62	7·346	21·0	41·0	9·0
10	6·60	7·346	23·0	42·0	—
11	8·40	6·813	13·5	40·5	—
12	10·03	6·990	13·5	40·5	—
13	7·15	7·000	17·0	40·0	—

[Similar results were obtained with undiluted plasma.]

* Measured by means of indicators and Walpole's colorimeter.

In order to elaborate the point still further experiments were instituted in which samples of different sera, previous to their being heated, were treated with acid and with alkali to ensure a gradation in the reaction of the sera from p_{H}^{+} 4·2 to 9·9.

Comparable results were obtained with each series of experiments. The typical results from one of these series have been embodied in Table IV and

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[1] These adjustments were made with the aid of indicators and Sörensen's solutions in Walpole's colorimeter. A certain amount of difficulty was experienced by me in the matching of colours in serum, especially so, with the indicators available, on the acid side. I subsequently ascertained that, owing to my defective matching, the real values of the hydrogen ion concentration varied from the values aimed at by amounts sufficient to account for the slight variations in the heat denaturation recorded in the Table.

TABLE IV.

Showing the influence of the hydrogen ion concentration of sera on the extent of the heat denaturation of the serum proteins induced during the prolonged heating of serum at a temperature of 57° for 6 hours.

Volume of normal acid (or equivalent) added to 100 cc. of the plasma diluted with one-third its volume of water	Volume of normal alkali (or equivalent) added to 100 cc. of the plasma diluted with one-third its volume of water	p_H^+ of the plasma determined by the electrical method	Appearance of the heated plasma	Percentage conversion of soluble into insoluble protein during the heating of the diluted plasma A	Percentage increase in the precipitability of the soluble proteins by 30 % of saturation with ammonium sulphate B	Percentage destruction of antitoxin during the heating process
—	6·55 cc. N . NH_3	9·945	clear	2·2	55·7	nil
—	4·92 ,,	9·910	,,	2·0	53·5	
—	3·275 ,,	9·789	,,	1·5	46·6	
—	2·62 ,,	9·477	,,	1·5	45·5	
—	1·96 ,,	9·304	,,	2·2	46·5	nil
—	1·31 ,,	8·870	,,	3·7	41·5	
—	0·65 ,,	8·249	clear liquid and slight deposit	2·2	36·8	
0·00 cc. N . HAc	0·00 ,,	7·430	,, ,, ,, ,,	3·3	22·3	nil
0·63 ,,	—	6·990	opalescent liquid and filtrate	5·2	10·2	
1·25 ,,	—	6·450	,, ,, ,, ,,	5·2	12·0	nil
1·87 ,,	—	5·921	,, ,, ,, ,,	3·7	13·1	
2·49 ,,	—	5·440	thin suspension, clear filtrate	6·3	13·1	
3·74 ,,	—	5·225	,, ,, ,, ,,	20·8	25·7	nil
2·50 cc. N . H_2SO_4	—	5·160	,, ,, ,, ,,	26·0	35·8	nil
4·17 cc. N . HAc	—	5·025	thick ,, ,, ,,	30·0	45·0	
2·52 cc. N . HCl	—	5·000	,, ,, ,, ,,	34·0	44·2	
4·99 cc. N . HAc	—	4·600	,, ,, ,, ,,	54·0	70·0	nil
3·60 cc. N . HCl	—	4·635	,, ,, ,, ,,	54·0	74·0	
3·60 cc. N . H_2SO_4	—	4·643	,, ,, ,, ,,	50·0	74·0	
6·74 cc. N . HAc	—	4·560	,, ,, ,, ,,	68·0	77·5	nil
4·40 cc. N . H_2SO_4	—	4·262	almost solid	98·0	not fluid enough to be measured	
4·36 cc. N . HCl	—	4·251	,, ,,	92·0		

With the sera ranging from p_H^+ 9·9 to 5·4 the extent of the changes recorded in *B* was a function of the time of heating, whereas with the more acid sera the change was practically completed within one hour's heating at the specified temperature.

have also been represented graphically in the curves in Fig. 2. Of the two, Curve 1 illustrates the changes in the solubility of the serum proteins during the heating of the serum, while Curve 2 indicates the accompanying changes in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate.

It was found that, with each of the sera examined, the extent of the changes involved was a function of the hydrogen ion concentration of the serum irrespective of the protein content: the use of undiluted serum or plasma gave rise to results similar to those obtained from experiments with plasma diluted with one-third its volume of water.

The appearance of the heated sera was characteristic throughout the ranges of acidity and alkalinity investigated.

It was found that the heated sera were clear when the reaction of the unheated serum had been more alkaline than p_H^+ 8·3. When the reaction had been between the limits p_H^+ 8·2 and 5·5 the heated sera were characterised by an opalescent appearance or by the presence of a slight deposit of protein. On the acid side of p_H^+ 5·5 the sera were converted, during the heating, into thin suspensions, the semi-solid consistency of which became more marked as the degree of acidity increased, until at p_H^+ 4·2 the serum became practically solid.

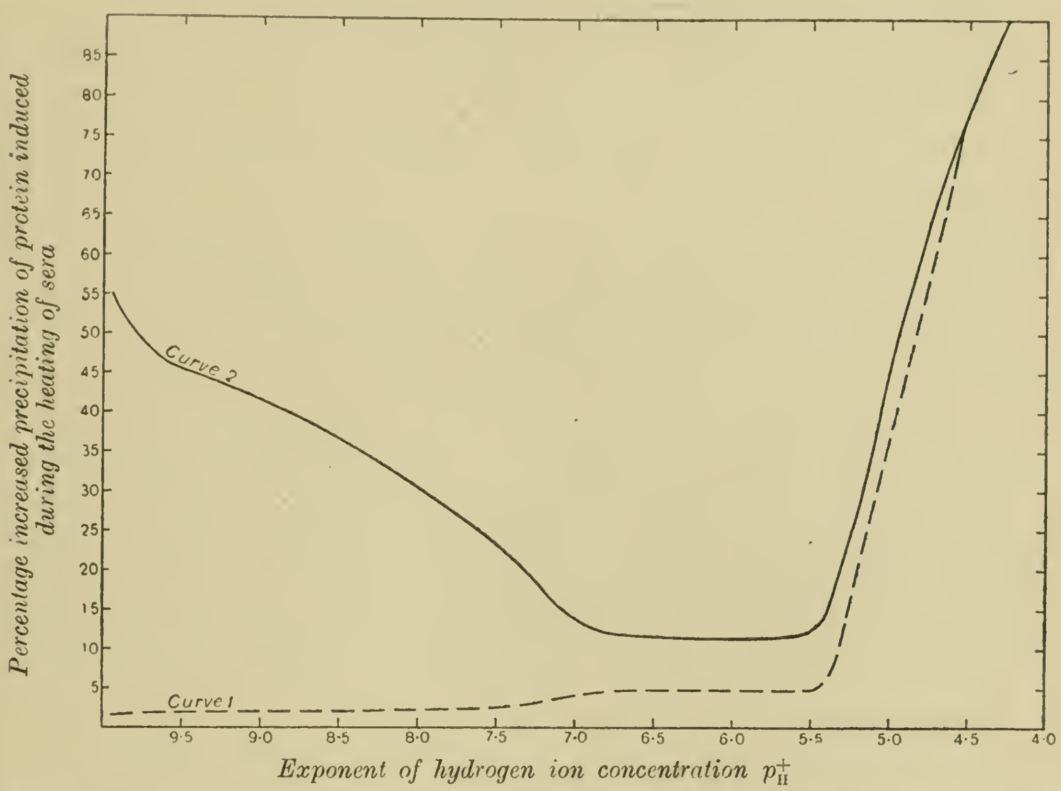

Fig. 2. The curve ——— represents the influence of changes in the reaction of sera on the percentage conversion of soluble into insoluble proteins induced during the heating of sera at 57° for 6 hours.

The curve represents the influence of changes in the reaction of sera on the percentage increased precipitability of the serum proteins by 30 % of saturation with ammonium sulphate induced during the heating of sera at 57° for 6 hours.

From a study of the data given in Table IV, Column A, it will be seen that the conversion of soluble into insoluble protein was of the order of 5 % or less in the sera of which the reaction lay between the ranges p_H^+ 9·9 and 5·5. On the acid side of p_H^+ 5·5 there was marked precipitation of the soluble proteins; thus at a hydrogen ion concentration of p_H^+ 5·4 it was of the order of 6 %, at p_H^+ 5·0 it was 30 %, while at p_H^+ 4·2 it was over 90 %.

From a consideration of Column B in the same table it will be seen that, irrespective of any accompanying conversion of soluble into insoluble protein, there was throughout the whole range investigated a marked increase in the precipitability of the serum proteins by 30 % of saturation with ammonium sulphate: the extent of the increase was a function of the reaction of the serum previous to its being heated.

This increased precipitability was least between the ranges p_{H}^{+} 5·4 and 7·0. On either side of these limits the rate of increase became apparent: the gradient for the increase was more gradual on the alkaline than on the acid side (Curve 2, Fig. 2). As the degree of alkalinity decreased from p_{H}^{+} 9·9 to 8·0 the increased precipitability gradually fell from 45 to 30 %. Between the ranges p_{H}^{+} 7·0 and 5·5 it was of the order of 10 to 15 %; on the acid side it rapidly rose from 25 % at p_{H}^{+} 5·2 to 90 % at p_{H}^{+} 4·2.

It was also interesting to note that, in all cases, there was no appreciable loss of antitoxin during the heating of sera the reaction of which had been adjusted to degrees of acidity or alkalinity within the limits dealt with in the accompanying tables, although under some conditions the extraction of the antitoxin from the heat-denaturated proteins proved a matter of difficulty.

From a study of the curves in Fig. 2 it is apparent that while on the alkaline side the configuration of Curve 1 is quite distinct from that of Curve 2, on the acid side of p_{H}^{+} 5·5 there is practical coincidence. There is, moreover, a marked similarity between the configuration of Curve 2 and that of the curve in Fig. 1. The extent of the increased precipitability on the alkaline side is not so marked in the latter as in the former, but on the acid side the curves, if superimposed, would be almost identical.

The question then arose as to whether the individuality of Curves 1 and 2 (Fig. 2) on the alkaline side indicated some radical difference between the changes respectively involved during the heating of alkaline and acid sera.

In order to elucidate this point samples of the clear heated alkaline sera which showed a 40 % increased precipitability by the sulphate were brought to an acidity of p_{H}^{+} 5·0. Now, had the changes involved during the heating of the alkaline sera been the same as those induced in the acid sera which showed a corresponding percentage increased precipitability, acidification of the clear alkaline sera should have changed the consistency of the latter to that of a thin suspension (*vide ante*, p. 29). A slight opalescence only was formed and the refractometer readings demonstrated that the quantity of protein thus precipitated was inappreciable. The opalescence therefore probably indicated that a small amount of heat changed protein had been kept in the disperse phase by the excess of alkali (*vide ante,* p. 26).

It was also noticed that the increased precipitability of the proteins of the heated alkaline sera was a function of the duration of the heating process. On the other hand the changes recorded in the acid sera were practically complete after one hour's heating at the specified temperature.

From these observations it is evident that the changes taking place during the heating of alkaline sera are of a different nature from those which occur during the heating on the acid side of the isoelectric point of the globulins. For this reason it is possible that if the heat denaturation be conducted on the acid side, there may be prepared concentrated products which will be less likely to give rise to the troubles of anaphylaxis and of serum sickness than the present products concentrated by methods involving a denaturation of the serum proteins by heating the slightly alkaline sera.

PRACTICAL APPLICATION OF THE RESULTS OBTAINED IN SECTIONS I, II AND III ABOVE.

The results of the investigation detailed above have led us to a better understanding of the causes underlying the irregularities encountered in the concentration of sera by methods involving the heating of the serum previous to the fractional precipitation of the serum proteins by ammonium sulphate.

We are finding that the production of clear and readily filterable end products can only be assured by controlling the reaction of the serum and of the serum mixtures unless certain expedients described below be resorted to.

An account of the investigation undertaken to overcome the causes of irregularities in the results obtained in (A) the Banzhaf (1913) method and later in (B) the method suggested by me [1916, 2] may be of service to those engaged in the concentration of antitoxic sera.

(A) *The regulation of the factors causing irregular results in the concentration of sera by the Banzhaf* (1913) *method.*

It seems probable that the difficulties in filtration so often encountered in this method are due to the presence of incompletely precipitated euglobulin and also of the small amount of protein denaturated during the heating of the serum.

As an outcome of the work recorded above it has been concluded that, with the published technique, there can be no guarantee for the complete elimination of these disturbing factors, and this for two reasons:

(1) That no cognisance has been taken of the reaction of the serum-ammonium-sulphate mixtures previous to their being heated.

(2) That there is no increased precipitation of the serum proteins during the heating of the serum mixtures.

It is generally recognised that rapid and clear filtration of the hot serum mixtures is one of the essential conditions for the production of readily filterable end products. In this laboratory it has been shown that, for the successful filtration of the hot serum mixtures, there must be assured the agglutination of the particles of precipitated euglobulin into aggregates sufficiently large for their retention on the surface of filter paper. From Tables I and II it is obvious that the success of this operation can only be guaranteed if the reaction of the mixtures be suitably adjusted.

It has been demonstrated that during the heating process advocated in this method the precipitating power of 30 % of saturation with ammonium sulphate is not appreciably enhanced. It is evident, therefore, that the small amount of euglobulin which must always escape precipitation with the First Fraction precipitate will appear in colloidal suspension in the final product. Unless the latter be allowed to stand for a considerable time until this colloidal suspension has settled as a deposit the unprecipitated protein proves a hindrance to filtration and is the cause of the marked clouding of so much of the antitoxic serum concentrated by this method.

The irregularities in the filtration of the serum mixtures can be obviated by an adjustment of the hydrogen ion concentration to the optimum required for the complete agglutination of the particles of "salted out" euglobulin and of the particles of protein denaturated during the heating of the serum. Good results can be obtained by adjusting the reaction to an acidity of about p_{H}^{+} 5·0. As however the determination of the hydrogen ion concentration of the sera by the electrical method is not always practicable in a routine laboratory, an endeavour is being made to work out a simple practical method by which the reaction can be adjusted by the use of suitable indicators.

In the meantime, pending the search for suitable and reliable indicators, it has been shown that the irregularities in the filtration of the serum mixtures can also be obviated by the simple expedient of adding 1·5 to 2 % of sodium chloride to the mixtures previous to their being heated.

It is important to know whether the salt improves the filtration of the hot serum mixtures by virtue of a specific action on the particles of precipitated protein or by an adjustment of the reaction of the medium more nearly to the desired optimum.

In order to elucidate this point duplicate samples of several plasmas were taken. To one of each of the duplicate pairs was added 1·5 to 2 % of sodium chloride. The hydrogen ion concentration of the duplicates was measured. The liquids were then made 30 % of saturation with ammonium sulphate and gradually heated to 61°.

From the results given in Table V it will be seen that the addition of sodium chloride decreases the alkalinity of the serum and of the serum mixtures. From a comparison with the data given in Table II, it is however obvious that the change in the hydrogen ion concentration induced by the addition of the salt is insufficient to account for the marked improvement in filtration and for the absence of an opalescent suspension in the filtrates.

TABLE V.

Showing the influence of the addition of sodium chloride to the serum on the hydrogen ion concentration of the serum and of the serum-ammonium-sulphate mixtures.

p_H^+ of the plasma diluted with one-third its volume of water = A	p_H^+ of A made 30 % of saturation with ammonium sulphate	Percentage of NaCl added to the diluted plasma	p_H^+ of the salted plasma = B	p_H^+ of B made 30 % of saturation with ammonium sulphate	Remarks on the filtrate from the hot serum-ammonium-sulphate mixtures from A	B
7·485	—	1·5	7·216	—	opalescent	clear
7·408	—	2	7·200	—	,,	,,
7·701	—	1·5	7·485	—	slightly opalescent	,,
7·701	—	4	7·485	—	,, ,,	,,
7·701	—	8	7·485	—	,, ,,	,,
7·347	—	2	7·223	—	opalescent	,,
—	7·250	1·5	—	7·010	,,	,,
—	7·220	2	—	7·000	,,	,,

It seems justifiable, therefore, to conclude that the improvement in filtration is induced by a specific action of the sodium chloride on the particles of precipitated protein whereby their satisfactory agglutination is assured.

Although the salt greatly improves the filtration of the serum mixtures, it cannot completely obviate the difficulties with the final product for it is powerless to ensure the complete precipitation of the euglobulin by 30 % of saturation with ammonium sulphate. Even in the presence of salt the desired increased precipitation of the serum proteins during the heating process does not take place.

In order to guarantee the elimination of euglobulin from the final products there were three courses open, viz.

(i) *The old method of extraction with brine.*

The Second Fraction precipitate containing the pseudo-globulin and antitoxin together with the small amount of attendant euglobulin and heat-denaturated protein was thrown into brine. The pseudo-globulin-antitoxin combination passed into solution while the euglobulin remained undissolved and was separated by filtration. The pseudo-globulin and antitoxin were precipitated by the addition of 0·25 % of glacial acetic acid to the clear filtrate. The precipitate was pressed and dialysed: the end product was crystal clear and its filtration presented no difficulty as it contained no trace of euglobulin or of heat-denaturated protein.

From the practical standpoint this method is not favoured as it involves a considerable amount of extra time and labour.

(ii) *A combination of the prolonged heating process of the Gibson-Banzhaf method with the shortened heating process of the Banzhaf* (1913) *method.*

By the adoption of this course it has been possible to obtain end products which, while showing an increased potency of from 8 to 10 times that of the original serum, do not exhibit the same tendency to form the cloudy deposit so often seen in the sera concentrated by Banzhaf's method. However, recent observations on the factors limiting the extent of the concentration of sera by the fractional precipitation methods at present in use [Homer, 1917], have demonstrated that there is a limit to which the concentration of sera can be carried. It was found that in all cases where the load of antitoxin attached to one gram of protein in the final product threatened to be greater than of the order of 25,000 units, the excess of units beyond this limit were transferred to the First Fraction precipitate.

From these observations it is evident that, for high grade sera (over 600 units per cc.), the above modification, yielding under ordinary circumstances a concentration of 8 to 10 times, cannot be used without apparent loss of antitoxin. The missing antitoxin can be recovered by extraction of the First Fraction precipitate with saturated salt solution.

To provide for such cases a third method of procedure has been employed, viz.

(iii) *The addition of substances such as phenol and its homologues to the serum mixtures previous to their being heated.*

Some years ago MacConkey [1914] observed that, during the heating of carbolised and of etherised sera there was a considerable loss of antitoxin. He subsequently found that the loss was negligible if 1·5 to 2 % of salt had previously been added to the serum.

In this laboratory it has also been demonstrated that, in the presence of salt, there is usually no appreciable loss of antitoxin during the concentration of carbolised sera by Banzhaf's method, if the temperature of the serum mixtures be not raised beyond 61°. At temperatures of 63° and 65° the loss becomes appreciable.

In the routine work, however, it has sometimes happened that, during the concentration of carbolised sera, there were appreciable losses of antitoxin even in the presence of salt. The records showed that in some of these cases the serum had been heated to a temperature of 65°, a procedure which in itself was sufficient to account for the loss. In other cases the records showed that during the concentration there had been removed more than 80 % of the total proteins of the original serum. With such a high removal of the serum proteins a certain proportion of the antitoxic units must be carried down with the First Fraction precipitate instead of with the Second Fraction precipitate. The antitoxin thus brought down with the First Fraction precipitate can only be extracted therefrom with great difficulty; hence the apparent and inexplicable loss of antitoxin under the conditions of the concentration.

It having been previously noticed that the heat-denaturation induced in the prolonged heating of sera to 57° is considerably increased by the addition of phenol, trikresol and cresylic acid to the serum, an experiment was made to ascertain whether the same phenomenon was experienced in the shortened heating process of Banzhaf's method. As this was found to be the case the investigation was carried further to ascertain whether the increased precipitation was due to changes in the hydrogen ion concentration caused by the addition of these substances.

In Table VI have been embodied data to illustrate the influence of phenol and cresylic acid on the precipitation of the serum proteins during the heating of the serum-ammonium-sulphate mixtures by the Banzhaf (1913) method. Similar results have been obtained by other workers in this laboratory from the use of trikresol, ether and chloroform.

From a study of the table it will be seen that while the addition of the above-mentioned substances had no appreciable effect on the hydrogen ion concentration of the sera, there was a marked increase in the precipitation of protein during the heating and at the same time a marked improvement in the filtration of the hot serum mixtures. The end products were characterised by their clearness and their ease of filtration; they contained no euglobulin.

TABLE VI.

Showing the increased precipitation of serum proteins induced by the addition of phenol and its homologues to the serum-ammonium-sulphate mixtures previous to their being subjected to the shortened heating process of the Banzhaf (1913) *Method.*

Percentage of phenol or its homologues added to the plasma diluted with one third its volume of water	$p_{\rm H}^{+}$ of the carbolised plasma measured by the electrical method	$p_{\rm H}^{+}$ of the carbolised plasma made 30 % of saturation with ammonium sulphate	Percentage conversion of soluble into insoluble protein during the heating process	Remarks on the filtration of the hot serum-ammonium-sulphate mixtures	Loss of antitoxin during the heating process
I. *Plasma containing* 8·74 % *of protein.*					
0·0 phenol	7·748	7·347*	6·3	slow, filtrate opalescent	nil
0·1 ,,	7·730	7·035†	16·5	more rapid, filtrate slightly opalescent	,,
0·2 ,,	7·730	7·034	23·0	rapid, filtrate clear	,,
0·3 ,,	7·730	7·085	24·0	,, ,, ,,	slight
0·4 ,,	7·730	7·085	26·0	,, ,, ,,	nil
0·5 ,,	7·730	7·061	33·0	,, ,, ,,	slight
II. *Plasma containing* 7·92 % *of protein.*					
0·0 phenol	7·460	—	2·0‡	slow, filtrate opalescent	
0·1 ,,	7·347	—	17·0	rapid, filtrate slightly opalescent	
0·2 ,,	7·347	—	24·4	rapid, filtrate clear	
0·4 ,,	7·347	—	27·9	,, ,, ,,	
0·5 ,,	7·347	—	30·8	,, ,, ,,	
III. *Plasma containing* 10·03 % *of protein.*					
0·0 cresylic acid	7·000	—	1·0	slow, filtrate opalescent	nil
0·35 ,, ,,	7·000	—	27·0	rapid, filtrate clear	,,
IV. *Plasma containing* 7·88 % *of protein.*					
0·0 cresylic acid	7·648	—	5·0	slow, filtrate opalescent	
0·30 ,, ,,	7·648	—	26·0	rapid, filtrate clear	

* $p_{\rm H}^{+}$ of the saturated solution of ammonium sulphate added to the plasma = 6·480.

† ,, ,, ,, ,, ,, ,, = 6·010.

‡ ,, ,, ,, ,, ,, ,, = 5·900.

As the addition of phenol, etc., to the serum mixtures previous to their being heated does not cause any alteration in the hydrogen ion concentration it is obvious that the improvement in filtration is due to the specific action of these substances on the proteins themselves, although the mode of action is different from that of sodium chloride. The latter merely serves to stimulate the aggregation of the particles of already precipitated protein, the former induce a considerable increase in the precipitation of the serum proteins by 30 % of saturation with ammonium sulphate, thereby assuring the complete precipitation of euglobulin at this stage. It is quite possible that the increased heat-denaturation of the serum proteins is brought about by an effect of these substances on the surface tensions of the colloidal particles of dissolved protein whereby their precipitability is considerably increased. Whatever may be the fundamental cause of this change the fact remains that the effect is so marked that, not only is the precipitating power of 30 % of saturation with ammonium sulphate considerably increased, but, in the cases investigated, the agglutination of the aggregates of the precipitated protein is so well defined that the reaction of the serum mixtures is of secondary importance. Moreover, the proteins involved do not seem to enter the intermediate ill-defined stage between the emulsoid and the suspensoid state which proves so great a hindrance to the filtration of the serum mixtures and of the final products.

From the data embodied in Table VI we see that, by the addition of phenol or its homologues, the heat-denaturation of the serum proteins can be regulated to the extent suitable for a particular serum.

For those who prefer to use the Banzhaf method this expedient is recommended until a simple routine method for the adjustment of the reaction of the serum mixtures previous to their being heated has been worked out. The judicious addition of 0·35 to 0·5 % of phenol or of 0·25 to 0·35 % of its homologues to the batches of plasma for concentration will result in the production of clear and readily filterable end products. If the addition of salt is also made a matter of routine, the concentration of the carbolised plasma or sera will not involve undue loss of antitoxin, although a considerable loss may result if the substances in question are added in amounts greater than those suggested.

(B) *The regulation of the factors causing irregular results in the concentration of sera by the more recent methods.* [Homer, 1916, 2.]

It has been found that there was slight trouble with the filtration of the end products from the concentration of sera in which the heat-denaturation had been less than 20 %, that is to say from the concentration of sera the reaction of which lay between the limits p_{H}^{+} 5·4 and 7·0. In these cases the end products contained a small amount of heat-denaturated protein in colloidal suspension.

The occurrence of this difficulty could be prevented by a preliminary adjustment of the serum, previous to its being heated, to a hydrogen ion concentration of about p_H^+ 7·8 to 8·0. It was inadvisable to increase the alkalinity further for the reasons given (*vide ante*, pp. 30, 31).

Trouble with the final filtration of end products from the concentration of sera of which the reaction fell within the limits p_H^+ 5·4 to 7·0 could also be obviated by the judicious addition of from 0·25 to 0·35 % of phenol or its homologues to the serum previous to the preliminary heating.

It is inadvisable to add the latter substances to more alkaline or to more acid sera than those falling between the aforesaid limits as there is the danger of thereby inducing so great an increased precipitability of the serum proteins that transference of antitoxin to the First Fraction precipitate will occur. Under these conditions there will be an apparent loss of antitoxin during the concentration as the extraction of the antitoxin thus carried down with the First Fraction precipitate is a matter of great difficulty.

It has also been found advisable to adjust the reaction of the serum-ammonium-sulphate mixtures previous to the second stage of the heating process (*ante*, p. 32). It may also be stated that at this stage of the heating the temperature of the serum mixtures is not raised beyond 59°.

From the consideration of the work detailed above it is evident that the reaction of the sera must be taken into account by those engaged in the concentration of antitoxic sera. On the reaction of the serum depends, not only the extent of the heat-denaturation of the serum proteins, but also the successful precipitation of the serum proteins by ammonium sulphate. Unless the reaction be regulated the necessary increased precipitation of the proteins cannot be ensured except by the addition of phenol, etc., to the serum. The latter course, in the hands of inexperienced workers may prove somewhat costly, as these substances favour the destruction of antitoxin.

Although the regulation of the reaction of the serum is proving of material value the possibility must not be lost sight of it being shown at some future date, that, in the concentration of antitoxic sera, there are other, hitherto, unsuspected factors which also influence the operations involved. In any case it is clear that the standardisation and regulation of a concentration process can only be attained by a thorough knowledge of the factors involved.

SUMMARY.

During the course of the investigation it has been demonstrated that:

(1) In the Banzhaf (1913) method the uncertainties in filtration are due to two causes:

(*a*) That no cognisance has been taken of the reaction of the serum.

(*b*) That as the precipitating power of 30 % of ammonium sulphate is not appreciably increased during the heating process, a certain amount of euglobulin escapes precipitation with the First Fraction precipitate and appears in colloidal suspension in the final product.

(2) The uncertainties in the filtration of the hot serum-ammonium-sulphate mixtures in the above method can be obviated by an adjustment of the hydrogen ion concentration. The filtration can also be improved by the addition of sodium chloride to the mixtures, but, in this case, the improvement is due to a specific action of salt on the globulins.

(3) The complete elimination of euglobulin can be assured by:

(*a*) An adjustment of the hydrogen ion concentration of the serum mixtures to the point at which the desired increased precipitation is assured.

(*b*) Brine extraction of the Second Fraction precipitate containing the pseudo-globulin-antitoxin combination together with the small amount of euglobulin which has escaped precipitation with the First Fraction precipitate.

(*c*) Subjecting the serum to a preliminary prolonged heating at a temperature of 57—58° whereby the precipitability of the serum proteins is considerably enhanced.

(*d*) The addition of organic substances such as phenol and its homologues, ether or chloroform which increase the precipitation of the serum proteins, presumably, by virtue of their effect on the surface tension of the protein aggregates in colloidal solution.

(4) The extent of the heat-denaturation of the serum proteins during the heating of serum at 57° for several hours is also influenced by the hydrogen ion concentration of the serum and can be controlled by the adjustment of the latter.

(5) The denaturation induced by heat in alkaline sera apparently does not involve the same type of change as that induced in acid sera.

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THE EFFECTS OF VACCINE SENSITISED WITH HOMOLOGOUS IMMUNE SERUM, AS COMPARED WITH THOSE OF A NON-SENSITISED VACCINE: AN EXPERIMENTAL STUDY.[1]

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Introduction.

Both active and passive immunity have certain disadvantages. With the latter, protection sets in very soon after inoculation, but lasts a very short time. The former, however, gives a lasting immunity, but the antibodies appear relatively late after inoculation, and the protected condition is preceded by a period of increased sensitiveness (negative phase). In order to counteract such objections, Besredka (1902 [4. 5. 7]) improved the method of sero-vaccination, which had been already recommended for this purpose, first by Lorenz (1892), and later by others (cf. Ficker, 1913 [20]). He used bacillary bodies (typhoid, cholera, and plague bacillus) which were first killed by heat and then treated with immune serum, the excess of which was removed by centrifuging. He claimed that this kind of vaccine was absolutely non-toxic for animals, that vaccinated animals obtained protection very soon after inoculation, with the appearance of large quantities of immune bodies in their serum, and that the immunity thus obtained lasted for months.

A. Marie (1902 [27]) introduced a sensitised anti-rabic vaccine prepared on the same principle. Then Dopter (1909 [19]) also applied this method to protect mice against dysentery, the results supporting Besredka's view. Later, Levy and Hamm (1909 [25]) recommended sensitised streptococci for the treatment of puerperal fever. Also Marxer (1911 [28]) proved that the same organism so sensitised gives the rabbit rapid immunity. Levy and Ao-ki (1910 [24]), comparing the effect of this kind of vaccine with that of the non-sensitised in the case of pneumococci, observed that the former gives the rabbit immunity with much more certainty and rapidity. Calmette and Guérin (1910 [15]) also sensitised the tubercle bacillus to inoculate cattle, and Meyer (1910 [33]) prepared a "Tuberkulose-Sero-Vaccin," treating human tubercle bacilli in the same way. Moreover, Garbat and Meyer (1911 [21]), having injected typhoid bacilli into rabbits intravenously, found that with the non-sensitised vaccine the animals

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suffer more from the injection than they do with the sensitised, and that their serum, though possessing higher agglutination and complement-fixation, shows lower bactericidal action *in vivo*.

While these authors were in favour of Besredka's principle, others held an entirely different view. In particular, Pfeiffer (1901 [36]) observed that by being loaded with amboceptors the cholera bacillus almost lost its immunising character, or at least its power of producing bacteriolytic antibodies. Pfeiffer and Bessau (1910 [37]) also recognised the same fact with the typhoid bacillus, and asserted that the method of immunising with sensitised bacilli is not practicable in this case. Lüdke (1911 [26]) treated the rabbit with sensitised dysentery bacilli without effect, for some animals, so treated twice, could not bear even a minimal lethal dose, and showed no increase of bactericidal power or of agglutinin in their serum. Further, Rowland (1912 [39]) asserts, in the case of the plague bacillus, that, as to toxicity and intensity of immunity, no difference is found between the sensitised and non-sensitised.

Metchnikoff and Besredka (1911–1912 [30. 31. 32]), having found that subcutaneous inoculation with the living sensitised typhoid bacilli did not, in the case of anthropoid monkeys, produce carriers, are said to have used the same vaccine in man for the prevention of typhoid without any disturbing effects. Broughton-Alcock (1912 [12. 13]) followed on the same lines, obtaining similarly good results. Then Ardin-Delteil, Négre, and Raynard (1912 [1]), and Boinet (1913 [11]), etc., applied this principle effectively to the treatment of many typhoid patients, the disease being shortened. Such treatment also was applied by Cohendy and Bertrand (1912 [16]), Broughton-Alcock (1913 [14]), Gordon (1913 [22]), Bertrand and Feigin (1913 [3]), and Cruveilhier (1913 [17. 18]) to various streptococcus and staphylococcus infections and gonorrhœal diseases, etc., with success on the whole. Further, Ardin-Delteil and his collaborators (1913 [2]) treated rabbits with living sensitised typhoid bacilli, and found a rapid and considerable rise in bactericidal action and complement-fixation, and yet no remarkable increase in agglutination. Lately Besredka (1913–1914 [8. 9. 10]), summarising the work of his followers, again recommended his own method, maintaining that whatever the nature of the virus, the sensitising gives it a new vaccinating character—the effect being sure, rapid, harmless, and lasting. Thus in France the sensitised vaccine is already being employed in practice, while in other countries it still remains for the most part the subject of theoretical investigation.

Before discussing the value of the living sensitised vaccine, it seems to me most important to ascertain whether the injected subject becomes a bacillary carrier or not. As this matter is beyond the scope of this thesis, I propose to limit my inquiry to the killed sensitised vaccine. Most investigators seem to admit that sensitised vaccine avoids the negative phase or at least shortens it, though on other points they are at variance. Accordingly, intent on settling some of these disputes as to the effects of sensitised vaccine, I undertook the following investigation, comparing it with the non-sensitised.

GENERAL DESCRIPTION.

I. The Bacteria used as Vaccine.

I chose the rabbit as subject, and adopted the *Bacillus pseudotuberculosis rodentium* (Pfeiffer) as the test organism. This organism readily attacks the rabbit and produces quite distinctive post-mortem appearances. The strain used showed characteristic properties with various culture media as follows:—

Table I.—*Characteristic Properties of* Bacillus pseudo-tuberculosis rodentium *shown with Various Culture Media.*

Medium.	Effect.	Medium.	Effect.
Glucose . . .	Af 1	Sorbite . . .	–
Lactose . . .	–	Glycerin . . .	Af 7.
Lævulose . . .	Af 1	Inulin . . .	–
Maltose . . .	Af 1	Litmus milk . .	Changes to alkaline after five days.
Saccharose . .	–	Malachite - green broth	Decolorised after three days.
Raffinose . . .	–	Broth . . .	Turbid.
Adonite . . .	Af 21 [1]	Gelatin . . .	No liquefaction.
Dulcite . . .	–	–	–
Isodulcite . .	Af 1	Peptone water . .	No trace of indol.

Af 1, Af 7, and Af 21 mean that a full acid reaction, but no gas, is produced in carbohydrate media after one, seven, and twenty-one days respectively.
– = Unaffected.

[1] Regarding the slow fermentation of adonite, cf. Kakehi (1916 [23]).

Taking Besredka's (1902 [4, 5]) method of making the vaccine, I had to investigate what degree of heat kills this organism, and in what

Table II.—*Resistance to Heat of the* Bacillus pseudo-tuberculosis rodentium.

Number of Minutes kept heated.	½ c.c. of Broth Culture, kept at 55° C. for the time indicated, was sown on Agar Plates.		½ c.c. of Broth Culture, kept at 53° C. for the time indicated, was sown on Agar Plates.	
	(1) Immediately after Heating.	(2) After Three Days' Incubation after Heating.	(1) Immediately after Heating.	(2) After Three Days' Incubation after Heating.
1 . . .	+ +	+ + +	...	...
2 . . .	+	+ + +	...	...
3 . . .	+	+ + +	...	...
5 . . .	+	+ + +	+ + +	+ + +
7 . . .	–	+	+ + +	+ + +
10 . . .	–	–	+ + +	+ + +
15 . . .	–	–	+ +	+ + +
20 . . .	–	–	+	+ + +
25 . . .	–	–	+	+ +
30 . . .	–	–	–	+
40 . . .	...	...	–	–
50 . . .	...	...	–	–

The table shows the number of colonies grown on agar plates after incubating for forty-eight hours.

– = None; + = scanty; + + = moderate; + + + = many colonies; ... = not tested.

time. Though many authors (namely, Poppé, 1913 [38], Messerschmidt and Keller, 1914 [29]) have described the resistance of this organism to heat, their statements disagree and seem to be far from exact.

Ten test tubes of ordinary size, each containing 5 c.c. of sterilised Loeffler's broth, are seeded with the bacilli and incubated for two days at 37° C. These tubes are simultaneously put into a big water-bath at the required temperature (53° or 55° C.). They are taken out of it one after another at the times given in Table II., counting from the instant when the temperature of their contents is identical with that of the bath, and are plunged at once into ice water to cool. (The temperatures of bath and tubes are identical after five to seven minutes.) Then from each tube ½ c.c. of its contents is poured on an agar plate, which is examined after incubating for forty-eight hours at 37° C. Each tube, however, is maintained in the incubator for three days more, and again ½ c.c. from each is cultivated for a similar period on another agar plate. Table II. shows that the bacilli can be killed within forty minutes at 53° C., and within ten minutes at 55° C. But to be absolutely sure that the bacilli are killed, I kept them at 55° C. for about fifteen minutes in all my experiments.

II. *Preparation of the Vaccines.*

First, the non-sensitised vaccine of this organism was made as follows. The emulsion of bacilli consisting of an agar-slope culture about twenty hours old, suspended in 5 c.c. of saline solution, is heated at 55° C. for fifteen minutes. Directly before heating, the number of bacilli contained in the emulsion was calculated by means of the Thoma-Zeiss counting slide. According to my experience, a 20-hour old agar-slope culture of this organism contains in most cases about 30,000 m.[1] bacilli, but in exceptional cases from 25,000 m. to 35,000 m. The emulsion containing a known quantity of killed bacilli was used as the non-sensitised (or ordinary) vaccine for the experiments, being diluted to any concentration required.

On the other hand, to make the sensitised vaccine, the necessary quantity of the emulsion prepared to contain exactly 5000 m. of killed bacilli in each cubic centimetre was mixed with the same quantity of rabbit immune serum, which had been produced by thrice repeated intraperitoneal injection of living bacilli, and which possessed an agglutination titre of 1 : 1280. The mixture was kept in the incubator for three to four hours, by which time the agglutination was almost complete. It was then kept in the cold overnight, to complete the sensitisation. Next morning the mixture was centrifuged for about an hour until no trace of bacilli was found in the supernatant fluid, which was carefully withdrawn by means of a capillary pipette. The sediment, after twice washing with saline solution, was suspended in fresh salt solution.

The two vaccines were always freshly prepared before every injection.

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[1] m. has been used as abbreviation for millions.

III. *Inoculation of the Rabbit.*

As there are often spontaneous cases of pseudo-tuberculosis in rodents, it is necessary to know whether the rabbits to be injected are quite healthy. Hitherto we have had no method of certain diagnosis of this disease. In my experience, estimating the agglutination titre of the serum, though in severe cases useful for diagnosis, does not always give constant results. Therefore I took the temperature of the rabbits twice every day, morning and evening, and weighed them every two or three days for seven or ten days before using them for the experiment. If the rabbits did not show any considerable variation of temperature and weight during that period, they were considered as healthy and used in the experiment.

In the next place, to avoid errors arising from individual variation as far as possible, I took rabbits of about the same size in each experiment and also injected the emulsion of bacilli in proportion to their weight. For the latter purpose the vaccine was prepared in such a concentration that each cubic centimetre contained the number of bacilli required per kilogram, and as many cubic centimetres of this are injected as there are kilograms of weight of the rabbit.

The vaccine was injected subcutaneously on the rabbit's abdomen, and to compare the effect of both kinds of vaccine completely, they were always in my experiments given simultaneously to an equal number of rabbits.

As a first injection of the vaccine I gave as large a dose as could be injected without provoking local or marked general reaction. Assuming that the sensitised vaccine, as many authors assert, is at least not much more toxic than the ordinary, I fixed the quantity of the dose at the amount usually given of the latter, as is shown in Table III.

Table III.—*Local Symptoms after the First Inoculation with the Non-Sensitised Vaccine.*

No.	Rabbit.		Bacilli injected per Kg.	Three Days later.		Seven Days later.	
	Sex.	Weight.		Weight.	Local Symptoms.	Weight.	Local Symptoms.
64	F.	1580	500 m.	1550	No trace.	1590	No trace.
65	F.	1560	1000 m.	1460	Slight trace.	1490	,,
68	M.	1820	1500 m.	1700	Slightly inflamed.	1780	Slightly infiltrated.
69	F.	1635	3000 m.	1520	Moderately inflamed.	1550	Abscess formation.

The result obtained shows that the infiltration and abscess formation *in loco* did not take place with absolute doses of under 1500 m., and the influence upon the weight was not remarkable with doses of under 500 m. per kg. The first injection consisted therefore of 500 m. per kg. or less.

Then seven days later this was followed by a second subcutaneous injection of twice, thrice, or four times as much as the first dose. Further, if necessary, a third, a fourth, and even a fifth injection was given after similar intervals. When in these succeeding injections, however, more than twice the previous dose was to be injected, to prevent any local reaction the dose was divided, and not more than twice the previous dose injected into any one place.

IV. Object of the Experiments.

In the first place, to study the degree of toxicity in the vaccine of both kinds, the ordinary and the sensitised, it is needful to pay special attention to the clinical symptoms of the injected rabbit, namely, local and general reaction. But as local reaction was purposely avoided by the smallness of each injection, only general reaction was observed. The following are the points in which the two vaccines are to be compared as to their general reaction:

1. The increase in the temperature of the rabbit after every injection with each vaccine.
2. The variation of a rabbit's weight after varying injections.

In the next place, to compare the intensity of immunity given by both kinds of vaccine, I determined:

1. The quantity of antibodies produced in the blood serum of the rabbit after injection, especially by agglutination and complement-fixation.
2. The resistance of the immunised rabbit against lethal doses of the living bacilli injected intravenously.

The results obtained are described in detail in the succeeding pages.

DETAILS OF OBSERVATIONS ON EFFECTS OF SENSITISED VACCINE COMPARED WITH THOSE OF NON-SENSITISED.

I. Comparison of the Influence of Both Vaccines upon the Temperature.

At first I have to examine the influence of the first injection of each vaccine, the ordinary and the sensitised, upon the temperature of the rabbits.

As first injections, doses of 200 m. and 500 m. bacilli were given per kilogram. For each series of the experiments twenty healthy rabbits were taken which were simultaneously injected, ten with ordinary vaccine, and ten with the sensitised. All the rabbits thus injected show temporarily an increase in temperature, and most of them get fairly high fever which reaches the maximum after two to four hours, exceptionally after six to eight hours, and decreases again to the normal after eight to twenty-four hours. Afterwards, their temperature was taken regularly twice every day for six days, and in every case was normal, as it had proved to be in the seven days preceding the experiment. Their rectal temperatures taken directly before and at intervals up to twenty-four hours after each injection, are tabulated in Tables IV. and V., and the results summarised in Table VI.

From the results described above we can draw the following conclusions:—

1. Both kinds of vaccine cause more or less increase of temperature in the rabbits within two hours after injection. But, comparing the average temperature every hour, the sensitised vaccine is generally found to have less reaction than the ordinary, though the difference is only slight (cf. Chart 1). Further, the same difference between the two vaccines is demonstrated by a comparison of the average highest temperatures reached after injection, and of the average increase in each case (see Table VI.).

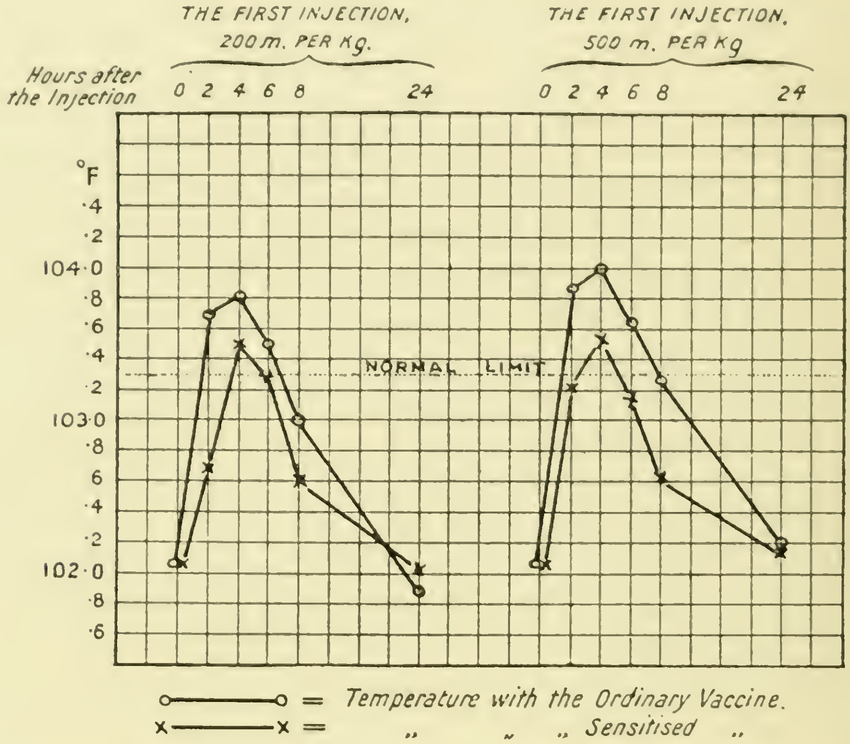


Chart 1.—Average temperature of the rabbits shown every two hours after the first injection of each kind of vaccine in quantities of 200 m. and 500 m. per kg.

2. The rabbits, when treated with the ordinary vaccine, all get more or less high fever after every injection. On the other hand, among those injected with the sensitised vaccine, when the dose was 500 m. per kg. one rabbit remained unfeverish throughout, while with a dose of 200 m. per kg. four so remained. Thus the sensitised

Table IV.—*Temperatures in ° Fahr. of the Rabbits after the First Subcutaneous Injection of* 200 *m. per Kg. of each kind of Vaccine.*

		Injected Rabbits.			Temperature taken before the Injection.	Temperature taken at Different Hours after the Injection.					Highest Temperature reached.	Amount of Increase.
		No.	Sex.	Weight (Grms.).		2 Hours.	4 Hours.	6 Hours.	8 Hours.	24 Hours.		
ORDINARY VACCINE.	1	118	M.	1270	102°·0	102°·6	103°·5	103°·3	102°·5	101°·3	103°·5	1°·5
	2	119	M.	1590	101°·7	103°·2	103°·3	103°·4	103°·0	101°·7	103°·4	1°·7
	3	120	M.	1200	102°·0	103°·6	103°·5	103°·2	102°·8	101°·7	103°·6	1°·6
	4	124	M.	1280	101°·9	105°·0	104°·6	104°·1	103°·5	101°·9	105°·0	3°·1
	5	125	M.	1330	101°·5	103°·3	103°·7	103°·7	103°·2	102°·0	103°·7	2°·2
	6	126	M.	1560	101°·4	102°·8	103°·9	103°·9	103°·5	101°·8	103°·9	2°·5
	7	130	F.	1840	102°·2	103°·5	103°·5	102°·8	102°·5	102°·1	103°·5	1°·3
	8	132	F.	1810	102°·7	104°·1	104°·0	103°·5	102°·6	102°·1	104°·1	1°·4
	9	136	F.	1810	102°·6	104°·5	104°·1	103°·5	103°·3	102°·3	104°·5	1°·9
	10	137	F.	1980	102°·6	104°·2	103°·9	103°·5	103°·1	102°·2	104°·2	1°·6
Average .			.	1567	102°·06	103°·68	103°·8	103°·49	103°·0	101°·91	103°·94	1°·88
SENSITISED VACCINE.	1	121	F.	1550	102°·8	103°·5	104°·5	104°·5	103°·6	102°·7	104°·5	1°·7
	2	122	F.	1330	101°·1	102°·8	103°·6	102°·8	102°·5	102°·1	103°·6	2°·5
	3	123	F.	1800	102°·0	102°·2	102°·9	102°·7	102°·0	101°·4	102°·9	0°·9
	4	127	F.	1390	102°·0	102°·1	103°·2	102°·9	102°·8	102°·5	103°·2	1°·2
	5	128	F.	1690	102°·5	102°·9	104°·2	103°·7	102°·5	102°·2	104°·2	1°·7
	6	133	M.	1710	101°·6	101°·8	102°·7	103°·1	102°·9	101°·5	103°·1	1°·5
	7	135	M.	1500	102°·0	103°·0	103°·1	102°·6	102°·5	101°·9	103°·1	1°·1
	8	138	F.	1450	101°·6	102°·3	103°·4	103°·1	102°·2	101°·7	103°·4	1°·8
	9	139	F.	1870	102°·8	103°·6	103°·9	103°·7	103°·0	102°·4	103°·9	1°·1
	10	142	M.	2130	102°·0	103°·0	103°·7	103°·6	102°·0	101°·9	103°·7	1°·7
Average .			.	1642	102°·04	102°·72	103°·52	103°·27	102°·6	102°·03	103°·56	1°·52

The experiment was performed on May 7, 1915.

TABLE V.—*Temperatures in ° Fahr. of the Rabbits after the First Subcutaneous Injection of 500 m. per Kg. of each kind of Vaccine.*

		Injected Rabbits.			Temperature taken before the Injection.	Temperature taken at Different Hours after the Injection.					Highest Temperature reached.	Amount of Increase.
		No.	Sex.	Weight (Grms.).		2 Hours.	4 Hours.	6 Hours.	8 Hours.	24 Hours.		
ORDINARY VACCINE.	1	157	F.	2840	101°·6	101°·3	102°·7	103°·5	102°·8	101°·9	103°·5	1°·9
	2	158	F.	2420	101°·4	103°·9	103°·3	102°·6	102°·2	101°·4	103°·9	2°·5
	3	159	M.	2220	102°·7	104°·6	105°·0	105°·4	104°·7	102°·2	105°·4	2°·7
	4	160	M.	2060	102°·3	104°·4	104°·4	103°·5	103°·1	102°·1	104°·4	2°·1
	5	161	F.	2880	102°·7	104°·8	104°·7	103°·3	103°·3	102°·7	104°·8	2°·1
	6	162	F.	2220	102°·1	104°·8	104°·7	103°·4	103°·3	102°·2	104°·8	2°·7
	7	163	F.	2780	101°·3	102°·8	103°·5	103°·7	104°·0	102°·6	104°·0	2°·7
	8	165	F.	2820	102°·0	104°·5	104°·2	103°·9	103°·9	102°·6	104°·5	2°·5
	9	166	F.	2220	101°·8	103°·5	103°·3	103°·3	102°·7	102°·2	103°·5	1°·7
	10	177	F.	2180	102°·6	104°·0	104°·2	103°·8	102°·5	102°·3	104°·2	1°·6
Average . . .				2494	102°·05	103°·86	104°·0	103°·64	103°·25	102°·22	104°·3	2°·25
SENSITISED VACCINE.	1	167	M.	1940	100°·9	102°·5	102°·8	103°·4	102°·7	101°·9	103°·4	2°·5
	2	169	M.	2560	102°·3	103°·0	103°·7	103°·0	102°·7	102°·3	103°·7	1°·4
	3	170	F.	2160	101°·5	103°·5	103°·1	102°·0	102°·0	101°·9	103°·5	2°·0
	4	171	F.	2260	102°·7	103°·9	104°·7	102°·7	102°·5	102°·2	104°·7	2°·0
	5	172	F.	2840	102°·3	103°·0	103°·6	103°·6	103°·2	102°·6	103°·6	1°·3
	6	173	F.	2280	102°·2	102°·8	103°·4	103°·8	103°·1	102°·3	103°·8	1°·6
	7	174	M.	2020	101°·8	102°·5	101°·9	103°·0	101°·8	102°·0	103°·0	1°·2
	8	176	M.	2040	102°·1	103°·0	103°·4	103°·3	102°·5	102°·0	103°·4	1°·3
	9	179	F.	2780	102°·5	103°·5	104°·2	103°·0	103°·0	102°·6	104°·2	1°·7
	10	180	F.	2120	102°·1	104°·5	104°·7	103°·7	102°·7	101°·9	104°·7	2°·6
Average . . .				2300	102°·04	103°·22	103°·55	103°·15	102°·62	102°·17	103°·8	1°·76

The experiment was performed on June 4, 1915.

TABLE VI.—*Comparison of the Increase of Temperature in ° Fahr. in Ten Rabbits after the First Injection of each kind of Vaccine.*

		Average of the Highest Temperature.	Average Amount of Increase.	Number of Feverish Rabbits (over 103°·3).
200 m. per kg.	Ordinary vaccine	103°·94	1°·88	10
	Sensitised ,,	103°·56	1°·52	6
	Difference	0°·38	0°·36	4
500 m. per kg.	Ordinary vaccine	104°·30	2°·25	10
	Sensitised ,,	103°·80	1°·76	9
	Difference	0°·50	0°·49	1

vaccine clearly produces not only a smaller relative increase of temperature, but also less frequent cases of fever than the ordinary.

3. The average of the highest temperatures, and that of the relative increase caused by an injection of 500 m. per kg. of sensitised vaccine are still a little lower than those produced by 200 m. per kg. of the ordinary. Yet it can hardly be concluded from this fact that the influence of a definite quantity of sensitised vaccine upon the temperature of the rabbits is less than that of about one-half that quantity of the ordinary, for the smaller the rabbit the more sensitive it seems to be to the vaccine (see Tables XI.–XV.). It can safely be assumed from the above results that the sensitised is the less toxic of the two vaccines.

Let us now compare the influence upon the temperature of the rabbit, after the second, third, and fourth injections of each kind of vaccine. Ten rabbits were taken to be injected with each kind of vaccine. These had been already injected with 200 m. per kg., as described in Table IV. Seven days after this first injection, an injection of 500 m. per kg. was made. A third and fourth injection of 1000 m. and 2000 m. per kg. was given after the same interval successively to each group of ten rabbits. The temperature of the rabbits thus injected rose one to two hours after the injections, and reached its maximum within four hours in most cases, and became normal after eight to twenty-four hours, except in one case, the reaction quite resembling that by the first injection. The temperatures taken directly before, and within twenty-four hours after each injection, are shown in Tables VII., VIII., and IX., and summarised in Table X. Chart 2 shows the averages at a glance.

In the case of these subsequent injections the difference in the number of the feverish rabbits under the two kinds of vaccine is not so pronounced as at the first injection. But the averages of the highest temperatures, and the average amounts of increase of temperature in the injected rabbits still show a certain difference, sufficient to

TABLE VII.

Rabbits, which seven days before had received doses of 200 m. per kilo of ordinary or sensitised vaccine, were given doses of 500 m. of the same kind of vaccine. Resulting temperatures shown in ° Fahr.

		Injected Rabbits.			Temperature before the Injection.	Temperature at Various Intervals after the Injection.							Highest Temperature reached.	Amount of Increase.
		No.	Sex.	Weight (Grms.).		2 Hours.	3 Hours.	4 Hours.	5 Hours.	6 Hours.	8 Hours.	24 Hours.		
ORDINARY VACCINE.	1	118	M.	1440	100°·8	102°·0	103°·0	103°·8	104°·0	103°·6	103°·6	102°·5	104°·0	3°·2
	2	119	M.	1660	101°·3	102°·9	103°·0	103°·7	103°·9	103°·7	102°·7	102°·3	103°·9	2°·6
	3	120	M.	1130	101°·6	102°·0	102°·8	103°·5	103°·9	103°·7	103°·6	101°·5	103°·9	2°·3
	4	124	M.	1160	101°·2	102°·2	103°·8	103°·9	103°·8	103°·0	102°·7	101°·0	103°·9	2°·7
	5	125	M.	1390	102°·0	103°·7	104°·1	104°·2	104°·3	103°·7	103°·0	101°·5	104°·3	2°·3
	6	126	M.	1590	102°·5	103°·0	103°·9	104°·2	103°·8	103°·3	103°·3	102°·9	104°·2	1°·7
	7	130	F.	1920	102°·2	103°·5	103°·6	103°·2	103°·0	102°·6	102°·2	102°·7	103°·6	1°·4
	8	132	F.	1830	101°·5	102°·9	103°·3	103°·4	103°·0	102°·6	102°·2	101°·5	103°·4	1°·9
	9	136	F.	1790	102°·0	103°·6	104°·2	104°·4	104°·3	103°·9	103°·7	102°·6	104°·4	2°·4
	10	137	F.	1810	102°·0	103°·2	103°·5	104°·1	103°·9	102°·9	102°·5	102°·4	104°·1	2°·1
Average .			.	1572	101°·71	102°·9	103°·52	103°·84	103°·79	103°·3	102°·95	102°·09	103°·97	2°·26
SENSITISED VACCINE.	1	121	F.	1660	102°·0	103°·4	104°·0	104°·4	104°·8	104°·5	104°·0	102°·5	104°·8	2°·8
	2	122	F.	1340	101°·4	102°·0	102°·9	102°·8	103°·2	102°·9	102°·0	101°·8	103°·2	1°·8
	3	123	F.	1690	100°·5	101°·0	101°·6	103°·1	102°·9	103°·0	102°·5	101°·4	103°·1	2°·6
	4	127	F.	1340	102°·0	102°·5	103°·7	103°·7	103°·2	102°·7	102°·4	102°·2	103°·7	1°·7
	5	128	F.	1600	101°·7	102°·0	103°·1	103°·4	103°·3	102°·9	102°·8	101°·1	103°·4	1°·7
	6	133	M.	1500	101°·3	102°·0	103°·2	103°·5	103°·5	103°·3	102°·5	101°·0	103°·5	2°·2
	7	135	M.	1570	102°·4	103°·2	103°·9	103°·8	103°·3	102°·9	102°·3	101°·5	103°·9	1°·5
	8	138	F.	1310	101°·7	102°·2	102°·8	103°·3	103°·2	102°·9	102°·6	102°·4	103°·3	1°·6
	9	139	F.	1910	102°·2	103°·7	104°·2	104°·5	104°·3	103°·5	102°·9	102°·6	104°·5	2°·3
	10	142	M.	2230	102°·0	102°·0	102°·9	103°·6	103°·4	102°·8	102°·5	102°·9	103°·6	1°·6
Average .			.	1615	101°·72	102°·5	103°·23	103°·61	103°·51	103°·14	102°·65	101°·94	103°·7	1°·98

This experiment was performed on May 14, 1915.

TABLE VIII.—*Temperatures after Third Injection of Vaccine.*

Temperatures in ° Fahr. after an injection of 1000 m. per kg. of each kind of vaccine in the rabbits which seven days before had received an injection of 500 m. per kg. of the same vaccine.

		Injected Rabbits.			Temperature before the Injection.	Temperatures at Various Intervals after the Injection.							Highest Temperature reached.	Amount of Increase.
		No.	Sex.	Weight (Grms.).		2 Hours.	3 Hours.	4 Hours.	5 Hours.	6 Hours.	8 Hours.	24 Hours.		
ORDINARY VACCINE.	1	118	M.	1120	101°·3	101°·8	102°·9	102°·4	102°·5	101°·9	102°·2	102°·0	102°·9	1°·6
	2	119	M.	1550	101°·8	103°·7	103°·9	103°·9	103°·8	104°·3	104°·0	102°·8	104°·3	2°·5
	3	120	M.	930	101°·1	101°·6	102°·6	102°·8	102°·9	102°·8	102°·9	101°·5	102°·9	1°·8
	4	124	M.	860	100°·8	101°·7	103°·7	103°·7	102°·8	102°·4	101°·7	101°·6	103°·7	2°·9
	5	125	M.	1270	102°·1	103°·5	104°·3	104°·2	103°·3	102°·8	102°·0	101°·5	104°·3	2°·2
	6	126	M.	1410	102°·5	103°·2	103°·5	103°·9	103°·6	102°·9	102°·8	102°·3	103°·9	1°·4
	7	130	F.	1830	102°·4	102°·7	103°·6	103°·0	102°·5	102°·3	102°·0	101°·8	103°·6	1°·2
	8	132	F.	1510	101°·3	103°·7	104°·2	103°·7	103°·6	103°·4	102°·6	102°·0	104°·2	2°·9
	9	136	F.	1650	102°·1	104°·0	104°·4	103°·7	103°·4	103°·3	102°·7	101°·8	104°·4	2°·3
	10	137	F.	1690	102°·4	104°·4	104°·8	104°·7	104°·0	102°·9	102°·1	102°·2	104°·8	2°·4
Average .			.	1382	101°·78	103°·03	103°·79	103°·6	103°·24	102°·9	102°·5	101°·95	103°·9	2°·12
SENSITISED VACCINE.	1	121	F.	1490	101°·7	102°·3	102°·8	102°·6	102°·4	102°·2	102°·0	101°·8	102°·8	1°·1
	2	122	F.	1120	101°·0	102°·5	102°·8	102°·1	101°·2	101°·6	101°·2	101°·4	102°·8	1°·8
	3	123	F.	1340	100°·7	102°·4	103°·0	103°·7	103°·0	103°·0	102°·0	102°·0	103°·7	3°·0
	4	127	F.	1370	101°·2	102°·9	103°·6	103°·7	103°·0	103°·0	102°·0	101°·7	103°·7	2°·5
	5	128	F.	1360	102°·1	103°·2	104°·0	103°·9	102°·9	102°·4	102°·4	102°·5	104°·0	1°·9
	6	133	M.	1450	101°·7	102°·0	103°·0	103°·4	102°·8	102°·5	102°·3	102°·3	103°·4	1°·7
	7	135	M.	1420	102°·2	103°·0	103°·2	103°·7	103°·7	103°·3	103°·0	102°·4	103°·7	1°·5
	8	138	F.	1300	101°·9	102°·5	103°·6	103°·3	103°·2	102°·4	102°·2	102°·0	103°·6	1°·7
	9	139	F.	1840	102°·3	102°·4	103°·9	103°·6	103°·1	102°·5	102°·3	102°·1	103°·9	1°·6
	10	142	M.	2140	101°·9	103°·2	103°·7	103°·4	103°·0	103°·1	102°·8	102°·2	103°·7	1°·8
Average .			.	1483	101°·67	102°·64	103°·36	103°·34	102°·83	102°·6	102°·22	102°·04	103°·53	1°·86

This experiment was performed on May 21, 1915.

TABLE IX.—*Temperatures after Fourth Injection of Vaccine.*

Temperatures in ° Fahr. after an injection of 2000 m. per kg. of each kind of vaccine in the same rabbits which had been seven days before treated with 1000 m. per kg. of the same vaccine.

		Injected Rabbits.			Temperature before the Injection.	Temperature at Various Intervals after the Injection.							Highest Temperature reached.	Amount of Increase.
		No.	Sex.	Weight (Grms.).		2 Hours.	3 Hours.	4 Hours.	5 Hours.	6 Hours.	8 Hours.	24 Hours.		
ORDINARY VACCINE.	1	118	M.	1140	100°·9	102°·5	103°·2	102°·2	101°·7	102°·1	102°·5	101°·9	103°·2	2°·3
	2	119	M.	1640	102°·0	103°·0	103°·5	103°·4	103°·3	103°·5	103°·2	102°·2	103°·5	1°·5
	3	120	M.	940	101°·1	103°·5	103°·0	102°·2	102°·1	101°·4	101°·5	101°·3	103°·5	2°·4
	4	124	M.	940	102°·4	104°·5	105°·2	104°·1	103°·5	103°·4	102°·6	102°·8	105°·2	2°·8
	5	125	M.	1340	102°·0	103°·0	104°·0	103°·0	102°·5	102°·3	101°·6	101°·7	104°·0	2°·0
	6	126	M.	1240	101°·6	102°·0	103°·8	103°·1	102°·0	101°·4	101°·8	101°·9	103°·8	2°·2
	7	130	F.	1880	102°·1	103°·4	103°·1	102°·3	101°·0	100°·9	100°·7	102°·2	103°·4	1°·3
	8	132	F.	1600	102°·1	103°·5	104°·4	103°·8	103°·5	103°·3	103°·0	102°·6	104°·4	2°·3
	9	136	F.	1720	101°·7	103°·3	103°·7	103°·1	103°·3	103°·3	103°·2	103°·0	103°·7	2°·0
	10	137	F.	1760	101°·4	103°·7	104°·7	103°·7	102°·6	101°·4	101°·0	102°·4	104°·7	3°·3
Average .			.	1420	101°·73	103°·24	103°·86	103·09	102°·55	102°·3	102°·11	102°·2	103°·94	2°·21
SENSITISED VACCINE.	1	121	F.	1420	102°·0	103°·5	104°·0	102°·9	103°·0	102°·8	102°·7	103°·5	104°·0	2°·0
	2	122	F.	1220	101°·2	103°·0	103°·4	102°·8	102°·5	102°·3	102°·0	102°·7	103°·4	2°·2
	3	123	F.	1520	100°·9	102°·2	102°·7	102°·2	102°·0	101°·9	101°·5	102°·0	102°·7	1°·8
	4	127	F.	1160	101°·6	103°·6	103°·8	103°·0	102°·8	102°·5	102°·1	102°·1	103°·8	2°·2
	5	128	F.	1540	102°·3	103°·0	103°·6	103°·2	103°·2	102°·7	102°·2	102°·7	103°·6	1°·3
	6	133	M.	1360	101°·7	103°·0	103°·2	103°·6	103°·3	102°·6	102°·0	101°·7	103°·6	1°·9
	7	135	M.	1440	101°·6	103°·0	103°·2	103°·2	103°·0	102°·4	101°·8	102°·2	103°·2	1°·6
	8	138	F.	1120	101°·7	103°·5	103°·7	104°·0	103°·7	103°·0	101°·9	102°·1	104°·0	2°·3
	9	139	F.	1320	101°·5	103°·2	103°·8	104°·1	103°·9	103°·0	102°·3	102°·5	104°·1	2°·6
	10	142	M.	2320	102°·1	103°·0	103°·2	103°·1	102°·6	102°·3	102°·0	102°·1	103°·2	1°·1
Average .			.	1442	101°·66	103°·1	103°·46	103°·21	103°·0	102°·55	102°·05	102°·36	103°·56	1°·9

This experiment (the fourth inoculation) was performed on May 28, 1915.

TABLE X.—*Comparison of the Increase of Temperature in ° Fahr. in Ten Rabbits after the repeated Injection of each kind of Vaccine.*

		Average of the Highest Temperature.	Average Amount of Increase.	Number of Feverish Rabbits (over 103°·3).
500 m. per kg.	Ordinary vaccine	103°·97	2°·26	10
	Sensitised ,,	103°·70	1°·98	8
	Difference	0°·27	0°·28	2
1000 m. per kg.	Ordinary vaccine	103°·90	2°·12	8
	Sensitised ,,	103°·53	1°·86	8
	Difference	0°·37	0°·26	0
2000 m. per kg.	Ordinary vaccine	103°·94	2°·21	9
	Sensitised ,,	103°·56	1°·90	7
	Difference	0°·38	0°·31	2

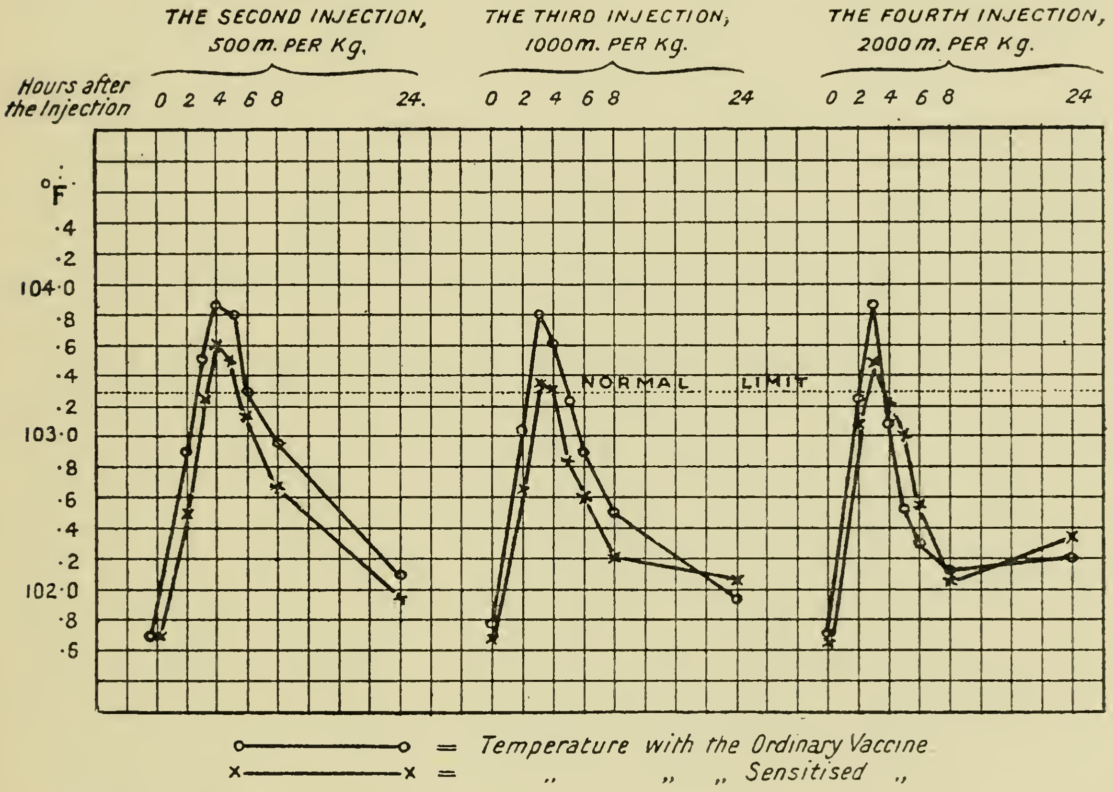


CHART 2.—Average temperature of the rabbits at various intervals after the injection of 500 m., 1000 m., and 2000 m. per kg. of each kind of vaccine respectively.

conclude that the sensitised vaccine is certainly less toxic than the ordinary at each repeated injection (see Table X.).

Further, I would emphasise the fact that my experience seems to be a little different from that of Garbat and Meyer (1911 [21]), who studied the temperature after intravenous injection with the two vaccines; for they say that the sensitised vaccine not only at the first, but also at succeeding injections produced a much higher and more rapid rise in temperature with a correspondingly rapid fall—phenomena which cannot be found in my cases. And though they assert that the general condition of health was better, and that the animals were livelier than with the non-sensitised, the cases that came under my notice showed no appreciable difference in these respects.

II. *Comparison of the Influence of Both Vaccines upon the Weight.*

As the weight of the rabbit varies greatly with different conditions, in this experiment care had to be taken to eliminate this variation as far as possible. Pregnant rabbits were rejected, and those used were always weighed fasting.

The experiments were of three kinds. In the first case a number of rabbits were inoculated twice, at intervals of seven days, first with 500 m., and secondly with 2000 m. per kg. In the second case, a number were inoculated three times at similar intervals with 500 m., 1500 m., and 5000 m. per kg. In the third case a third group were inoculated five times with 200 m., 500 m., 1000 m., 2000 m., and 5000 m. per kg. at intervals of seven days as before.

In the case of the first injection, 200 m. per kg. of either kind of vaccine does not produce any remarkable change in the rabbits' weight. However, when the dose of 500 m. per kg. is injected, in most cases a slight decrease of the weight can be observed after two or three days, the decrease being similar in the case of sensitised and non-sensitised vaccines; after five to seven days the weight remains almost the same as after two to three days (see Table XI.).

In the case of the succeeding injections, the variation in weight follows the same lines as after the first injection of 500 m., and the decrease in weight is usually directly proportional to the increase of the dose. In the case of the last injection the rabbits were weighed also after ten days, when in most cases they show a slight increase on the eighth day's weight, due perhaps to their recovery. But very sensitive rabbits still show progressive decrease of weight after the eighth day, and sometimes die of exhaustion. In my experience, however, no sudden or anaphylactic death occurred. In every case of death a careful post-mortem examination revealed no complicating disease, but confirmed my view that death was due to the inoculation.

The results shown in the Tables XI., XII., XIII., and XIV., and summarised in Table XV., give us the following conclusions:—

1. In each series of experiments, the average percentage of decrease in weight ten days after the last injection as compared with the original weight is relatively low in the case of the sensitised vaccine. On this point, a remarkable difference between the action of the two vaccines is observed, especially in the case of animals five times injected.

2. Further, the whole number of the rabbits which decreased in weight was found to be slightly higher with the ordinary vaccine than with the sensitised, and the number of those that lost over 10 per cent. in weight was very much greater with the former.

TABLE XI.—*Weight after First Injection with each Vaccine.*

		Rabbits injected with 200 m. per Kg.		Weight of Rabbits (Grms.).			Difference between the First and Last Weight.	Rabbits injected with 500 m. per Kg.		Weight of Rabbits (Grms.).			Difference between the First and Last Weight.
		No.	Sex.	Before the Injection.	Four Days after the Injection.	Seven Days after the Injection.		No.	Sex.	Before the Injection.	Four Days after the Injection.	Seven Days after the Injection.	
ORDINARY VACCINE.				7/v	11/v	14/v				4/iii	8/iii	11/iii	
	1	118	M.	1270	1320	1400	+130	41	F.	2595	2370	2400	−195
	2	119	M.	1590	1620	1660	+ 70	42	F.	2575	2490	2520	− 55
	3	120	M.	1200	1160	1130	− 70	50	M.	2420	2270	2195	−225
	4	124	M.	1280	1210	1160	−120	54	M.	2525	2480	2500	− 25
	5	125	M.	1330	1380	1390	+ 60	60	M.	2350	2350	2360	+ 10
										1/iv	5/iv	8/iv	
	6	126	F.	1560	1600	1590	+ 30	82	F.	1850	1880	1770	− 80
	7	130	F.	1840	1880	1920	+ 80	83	F.	2030	1940	1980	− 50
	8	132	F.	1810	1820	1830	+ 20	84	F.	1890	1890	1850	− 40
	9	136	F.	1810	1800	1790	− 20	85	F.	1790	1590	1680	−110
	10	137	F.	1980	1860	1810	−170	94	F.	1740	1770	1780	+ 40
Average . . .				1567	1565	1568	+ 1	...	...	2177	2103	2104	− 73
SENSITISED VACCINE.				7/v	11/v	14/v				4/iii	8/iii	11/iii	
	1	121	F.	1550	1695	1660	+110	44	M.	2650	2600	2620	− 30
	2	122	F.	1330	1310	1340	+ 10	55	F.	2530	2530	2560	+ 30
	3	123	F.	1800	1720	1690	−110	58	F.	2540	2380	2340	−200
	4	127	F.	1390	1380	1340	− 50	62	F.	2425	2350	2420	− 5
	5	128	F.	1690	1670	1600	− 90	63	M.	2550	2330	2360	−190
										1/iv	5/v	8/iv	
	6	133	M.	1710	1620	1500	−210	86	F.	1680	1610	1620	− 60
	7	135	M.	1500	1560	1570	+ 70	87	F.	1580	1550	1560	− 20
	8	138	F.	1450	1260	1310	−140	88	F.	1850	1710	1710	−140
	9	139	F.	1870	1980	1910	+ 40	89	F.	1690	1630	1650	− 40
	10	142	M.	2130	2210	2230	+100	90	M.	1970	1930	1910	− 60
Average . . .				1642	1641	1615	− 27	...	...	2147	2062	2075	− 72

Note (1).—7/v, etc., indicate the day and the month of inoculation.

TABLE XII.—*Weight of the Rabbits injected Twice with each Vaccine.*

	Rabbits injected with Ordinary Vaccine.		Weight of Rabbits (Grms.).			Difference between the First and Last Weight.	Percentage of Decrease on the Average.	Rabbits injected with Sensitised Vaccine.		Weight of Rabbits (Grms.).			Difference between the First and Last Weight.	Percentage of Decrease on the Average.
	No.	Sex.	Before the First Injection (500 m. per Kg.).	Seven Days after the First Injection.	Ten Days after the Second (last) Injection (2000 m. per Kg.).			No.	Sex.	Before the First Injection (500 m. per Kg.).	Seven Days after the First Injection.	Ten Days after the Second (last) Injection (2000 m. per Kg.)		
			4/iii	11/iii	21/iii					4/iii	11/iii	21/iii		
1	41	F.	2595	2400	2350	-245	..	44	M.	2550	2620	2570	+20	..
2	42	F.	2575	2520	2000	-575	..	55	F.	2530	2560	2580	+50	..
3	49[1]	M.	2505	2165	..	..	..	56	F.	2300	2200	2120	-180	..
4	50	M.	2420	2195	1980	-440	..	58	F.	2540	2340	2480	-60	..
5	54	M.	2525	2500	2490	-35	..	62	F.	2425	2420	2210	-215	..
6	60	M.	2350	2360	2460	+110	..	63	M.	2550	2360	2540	-10	..
	Average . .		2493	2395	2256	-237	9·5	..	..	2482	2417	2417	-65	2·6
			4/vi	11/vi	21/vi					4/vi	11/vi	21/vi		
1	158	F.	2420	2300	2200	-220	..	167	M.	1940	1980	2040	+100	..
2	159	M.	2220	2260	1860	-360	..	171	F.	2260	2260	2340	+80	..
3	160	M.	2060	2180	1980	-80	..	173	F.	2280	2140	1980	-300	..
4	162	F.	2220	1980	2060	-160	..	174	F.	2020	2060	1820	-200	..
5	164[1]	F.	2000	1900	..	..	..	176	M.	2040	2020	1880	-160	..
6	166	F.	2220	2160	2040	-180	..	..	M.	..	..	..	..	..
	Average . .		2228	2176	2028	-200	9·0	..	..	2108	2092	2012	-96	4·6
			2/vii	9/vii	19/vii					2/vii	9/vii	19/vii		
1	151	M.	1700	1620	1300	-400	..	156	F.	2170	2010	2120	-50	..
2	184	F.	2160	2240	2080	-80	..	178	F.	1740	1620	1360	-380	..
3	191	F.	1710	1620	1240	-470	..	182	F.	1920	1860	1660	-260	..
4	192	F.	1750	1740	1340	-410	..	185	F.	1800	1740	1880	+80	..
5	195	F.	1820	1780	1480	-340	..	193	F.	1800	1760	1460	-340	..
6	..	..	..	..	..	..	..	194[1]	F.	1600	1520	..	..	..
	Average . .		1828	1800	1488	-340	18·6	..	..	1886	1752	1696	-190	10·1

[1] *Note* (2).—Rabbits Nos. 49, 164, 194, etc., which have the last two or three columns blank, died of the injection, and these are disregarded when calculating the averages.

TABLE XIII.—*Weight of the Rabbits injected Thrice with each Vaccine.*

	Rabbits injected with Ordinary Vaccine.		Weight of Rabbits (Grms.).				Difference between the First and Last Weight.	Percentage of Decrease on the Average.	Rabbits injected with Sensitised Vaccine.		Weight of Rabbits (Grms.).				Difference between the First and Last Weight.	Percentage of Decrease on the Average.
	No.	Sex.	Before the First Injection (500 m. per Kg.).	Seven Days after the First Injection.	Seven Days after the Second Injection (1500 m. per Kg.).	Ten Days after the Third (last) Injection (5000 m. per Kg.).			No.	Sex.	Before the First Injection (500 m. per Kg.).	Seven Days after the First Injection.	Seven Days after the Second Injection (1500 m. per Kg.).	Ten Days after the Third (last) Injection (5000 m. per Kg.).		
			1/iv	8/iv	15/iv	25/iv					1/iv	8/iv	15/iv	25/iv		
1	82	F.	1850	1770	1790	1620	– 230	...	86	F.	1680	1620	1630	1490	– 190	...
2	83	F.	2030	1980	1990	1970	– 60	...	87	F.	1580	1520	1590	1370	– 210	...
3	84	F.	1890	1850	1870	1800	– 90	...	88	F.	1850	1710	1800	1740	– 110	...
4	85	F.	1790	1680	1610	1520	– 270	...	89	F.	1690	1650	1680	1600	– 90	...
5	92[1]	F.	1640	1690	1610	...	...	...	90	M.	1970	1910	2000	2070	+100	...
6	94	F.	1740	1780	1790	1520	– 220	...	91[1]	M.	1850	1680	1520	...	...	...
Average .			1860	1812	1810	1686	– 174	9·3	...	...	1754	1682	1740	1654	– 100	5·7
			4/vi	11/vi	18/vi	28/vi					4/vi	11/vi	18/vi	28/vi		
1	157	F.	2840	2820	2880	2880	+ 40	...	169	M.	2360	2320	2360	2250	– 110	...
2	161	F.	2880	2880	2880	2750	– 130	...	170	F.	2160	2180	2240	2180	+ 20	...
3	163	F.	2780	2680	2700	2780	0	...	172	F.	2840	2780	2740	2720	– 120	...
4	165	F.	2820	2720	2800	2720	– 100	...	179	F.	2880	2860	2960	2950	+ 70	...
5	177	F.	2480	2420	2380	2240	– 240	...	180	F.	1920	1840	1820	1780	– 140	...
Average .			2760	2704	2728	2674	– 86	3·1	...	...	2432	2396	2424	2376	– 56	2·3

[1] See Note (2), Table XII.

TABLE XIV.—*Weight of the Rabbits injected Five Times with each Vaccine.*

	Rabbits injected with Ordinary Vaccine.		Weight of Rabbits (Grms.).				Difference between the First and Last Weight.	Percentage of Decrease on the Average.	Rabbits injected with Sensitised Vaccine.		Weight of Rabbits (Grms.).				Difference between the First and Last Weight.	Percentage of Decrease on the Average.
	No.	Sex.	Before the First Injection (200 m. per Kg.).	Seven Days after the Second Injection (500 m. per Kg.).	Seven Days after the Fourth Injection (2000 m. per Kg.).	Ten Days after the Fifth (last) Injection (5000 m. per Kg.).			No.	Sex.	Before the First Injection (200 m. per Kg.).	Seven Days after the Second Injection (500 m. per Kg.).	Seven Days after the Fourth Injection (2000 m. per Kg.).	Ten Days after the Fifth (last) Injection (5000 m. per Kg.).		
			7/v	21/v	4/vi	14/vi					7/v	21/v	4/vi	14/vi		
1	118	M.	1270	1120	1260	1130	−140	...	121	F.	1550	1490	1640	1650	+100	...
2	118	M.	1590	1550	1630	1660	+ 70	...	122	F.	1330	1120	1340	1320	− 10	...
3	120[2]	M.	1200	930	880	...	...	...	123	F.	1800	1340	1440	1640	−160	...
4	124	M.	1280	860	1080	1040	−240	...	127	F.	1390	1370	1420	1440	+ 50	...
5	125	M.	1330	1270	1440	1400	+ 70	...	128	F.	1690	1360	1580	1620	− 70	...
6	126[2]	M.	1560	1410	...	...	...	...	133[2]	M.	1710	1450	...	...	...	...
7	130	F.	1840	1830	1820	1760	− 80	...	135	M.	1500	1420	1560	1440	− 60	...
8	132	F.	1810	1510	1500	1560	−250	...	138	F.	1450	1300	1360	1250	−200	...
9	136	F.	1810	1650	1780	1600	−210	...	139[1]	F.	1870	1840	1080	940	...	...
10	137	F.	1980	1690	1620	1520	−460	...	142	M.	2130	2140	2240	2160	+ 30	...
Average .			1614	1435	1516	1459	−155	9·6	...	...	1605	1443	1573	1565	− 40	2·5
			14/vi	28/vi	12/vii	22/vii					14/vi	28/vi	12/vii	22/vii		
1	131[2]	F.	2420	2370	...	...	...	...	129[2]	F.	2360	2180	...	...	...	...
2	150	M.	2450	2460	2380	2260	−190[1]	...	186	F.	2890	2940	2940	2840	− 50	...
3	183	F.	2780	2640	2660	2580	−200	...	189	F.	2620	2560	2480	2500	−120	...
4	187	M.	2460	2500	2460	2180	−280	...	190	F.	2780	2780	2820	2600	−180	...
5	188	M.	2720	2660	2640	2580	−140	...	197	M.	2560	2760	2840	2720	+160	...
Average .			2603	2565	2535	2400	−203	7·8	...	...	2713	2760	2770	2665	− 48	1·7

[1] *Note* (3).—Rabbit 139 was pregnant and was delivered during the experiment; it was, therefore, not reckoned in forming the average.

[2] *Note* (4).—For the sake of simplicity of tabulation the weights seven days after the first and seven days after the third injection have been omitted.

TABLE XV.—*Comparison of the Variation in Weight of the Injected Rabbits in each Series.*

Whole Number of Injected Rabbits.		Number of Rabbits without Decrease in Weight.	Number of Rabbits with Decreased Weight.				Number of Rabbits that died of the Inoculation.
			Under 5 per cent.	Between 5–10 per cent.	Over 10½ per cent.	Total.	
Twice injected	O 17	1	3	4	7	14	2
	S 17	4	4	4	4	12	1
Thrice injected	O 11	2	4	1	3	8	1
	S 11	3	2	3	2	7	1
Five times injected	O 15	2	1	3	6	10	3
	S 14	4	5	2	1	8	2

O = Ordinary vaccine.
S = Sensitised vaccine.

3. Regarding death caused by injection, it occurs more frequently with the ordinary vaccine. But the difference in number with the two kinds does not warrant the pronounced distinction between their toxicity which was made by Garbat and Meyer.

4. With all different kinds of injection, the rabbits of small size (under 2000 grms.) show a much higher percentage of decrease in their weight than the bigger ones, so that the smaller the rabbit the more sensitive it appears to be to the vaccine. In this case, again, the ordinary vaccine seems to have a greater effect than the sensitised.

Thus all the above results show that the sensitised vaccine, though not atoxic, is certainly less toxic than the ordinary, as has been already suggested in the case of temperature tests, yet the difference in toxicity is not very great. We can assume, however, with some probability, that, if the dose of the vaccine is gradually diminished, a point can be reached where the sensitised vaccine is no longer toxic, while the ordinary still shows some reaction, owing to the difference in their toxicity. Still, as the endotoxin is probably more or less extracted by the serum and saline solution from the bacillary bodies in the process of sensitising and washing (Besredka, 1906 [6]; Pfeiffer and Bessau, 1910 [37]; and others), it must be added that the decrease in toxicity of sensitised vaccine need not be attributed merely to loading the bacilli with amboceptors.

III. *Comparison of the Production of Antibodies by the Two Vaccines.*

The serum of the rabbits injected with each vaccine is here described only with reference to its power to agglutinate the homologous bacilli and its capacity to deviate complement, when conjoined with this organism. The bactericidal action of the serum was also tested by the common method of sowing on agar plates, but the result was not sufficiently pronounced with any serum, as Noon (1909 [35]) also found. In my experiments, even 0·1 c.c. of heated serum (derived from the rabbit injected thrice) in 1·5 c.c. could not kill after four hours a total of 3000 bacilli contained in that solution, together with 0·05 c.c. guinea-pig complement.

1. Agglutination.

To 1 c.c. of successive dilutions of the unheated fresh rabbit serum was added 1 c.c. of a suspension of the living bacilli containing about 1500 m. per cubic centimetre. After careful mixing, the tubes were incubated at 37° C. for three to four hours, when almost complete agglutination occurred, and then left overnight at room temperature. The results, read macroscopically after twenty-four hours, appear in the tables.

First, two groups of rabbits were chosen, each consisting of five animals, which were twice injected at intervals of seven days with 500 m. and 2000 m. per kilogram of each vaccine. Immediately before each injection, and two, five, and ten days after the second, the same quantity of the serum of each rabbit in the group was taken and mixed. The agglutinating power of each mixture was tested (see Table XVI.).

Table XVI.—*Agglutination shown in Various Intervals by the Rabbits injected Twice.*

Date of Serum.		Serum Dilution.								Control.
		1 : 20	40	80	160	320	640	1280	2560	
ORDINARY VACCINE.	Before the first injection.	–	–	–	–	–	–	...	...	–
	Seven days after the first injection.	+ +	+ +	±	–	–	–	–	–	–
	Two days after the second injection.	+ + +	+ +	+ +	+	–	–	–	–	–
	Five days after the second injection.	+ + +	+ + +	+ + +	+ + +	+ +	+ +	–	–	–
	Ten days after the second injection.	+ + +	+ + +	+ + +	+ + +	+ +	+	–	–	–
SENSITISED VACCINE.	Before the first injection.	–	–	–	–	–	–	...	...	–
	Seven days after the first injection.	+	–	–	–	–	–	...	...	–
	Two days after the second injection.	+ +	+	–	–	–	–	...	...	–
	Five days after the second injection.	+ + +	+ +	+	–	–	–	...	...	–
	Ten days after the second injection.	+ + +	+ +	+	–	–	–	...	...	–

– = No ; ± = doubtful ; + = partial ; + + = marked ; + + + = almost complete or complete agglutination.

In Table XVI. the sensitised vaccine is observed in every case to be incomparably less capable than the ordinary of producing agglutinin. For seven days after the first injection and two days after the second, the amount produced by sensitised vaccine was one-fourth the amount of that produced by non-sensitised, and only one-eighth at five to ten days after the second. Further, the production of agglutinin is found to vary with the time in a similar way in each series, with the titre highest five to ten days after the second injection.

Next, the agglutination of the sera obtained by two other methods of injection was tested. The five rabbits in each group were injected successively with 500 m., 1500 m., and 5000 m. per kilogram of each kind of vaccine, and as another kind of injection, with 200 m., 500 m., 1000 m., 2000 m., and 5000 m. Seven days after the last injection the serum of each rabbit was taken and separately tested. For results, see Tables XVII. and XVIII. respectively.

TABLE XVII.—*Agglutination of the Sera produced by Three Successive Injections.*

No. of Serum.		Serum Dilution.										Control.
		1 : 20	40	80	160	320	640	1280	2560	5120	10,240	
ORDINARY VACCINE.	82	+ + +	+ + +	+ + +	+ + +	+ +	+	–	–	–	–	–
	83	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+ +	±	–	–	–
	84	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+	–	–	–	–
	85	+ + +	+ + +	+ + +	+ + +	+ +	+	–	–	–	–	–
	94	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+ +	±	–	–	–
SENSITISED VACCINE.	86	+ +	+ +	+	–	–	–	–	–	...	...	–
	87	+ + +	+ + +	+ + +	+ +	±	–	–	–	...	...	–
	88	+	+	–	–	–	–	–	–	...	...	–
	89	+ + +	+ +	±	–	–	–	–	–	...	...	–
	90	+ + +	+ +	+	+	–	–	–	–	...	...	–

Explanation of symbols as in Table XVI.

Though in each group the agglutination is found to vary with the different rabbits owing to individual peculiarities, every serum produced by the sensitised vaccine showed far weaker action than that given by the non-sensitised in the case of either kind of injection. It is a noticeable point that the sera derived from the sensitised vaccine seem to differ greatly in agglutinating power. Similar results will be found in the tables in the next chapter, showing the lowest active dilution for agglutination of other sera. Quoting the theory of Neisser and Lubowski (1909[34]) who studied the agglutination after injection with agglutinated bacteria, the explanation of this fact perhaps may be that animals of high agglutination, when injected subcutaneously with the sensitised vaccine, have the power of breaking the attraction between agglutinin and bacillary receptors, the free bacillary receptors then stimulating the production of much more agglutinin.

TABLE XVIII.—*Agglutination of the Sera produced by Five Successive Injections.*

	Number of Serum.	Serum Dilution.										Control.
		1 : 20	40	80	160	320	640	1280	2560	5120	10,240	
ORDINARY VACCINE.	118 .	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+ +	+	±	−	−
	119 .	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	±	−	−	−	−
	124 .	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+	±	−	−	−
	125 .	+ + +	+ + +	+ + +	+ + +	+ +	+	−	−	−	−	−
	130 .	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+	−	−	−	−
SENSITISED VACCINE.	121 .	+ + +	+ + +	+	+	±	−	−	−	...	...	−
	122 .	+ + +	+ + +	+ + +	+ +	+	−	−	−	...	...	−
	123 .	+ + +	+ +	−	−	−	−	−	−	...	...	−
	127 .	+ + +	+ + +	+ +	±	−	−	−	−	...	...	−
	128 .	+ + +	+ + +	+ +	+	−	−	−	−	...	...	−

Explanation of symbols as in Table XVI.

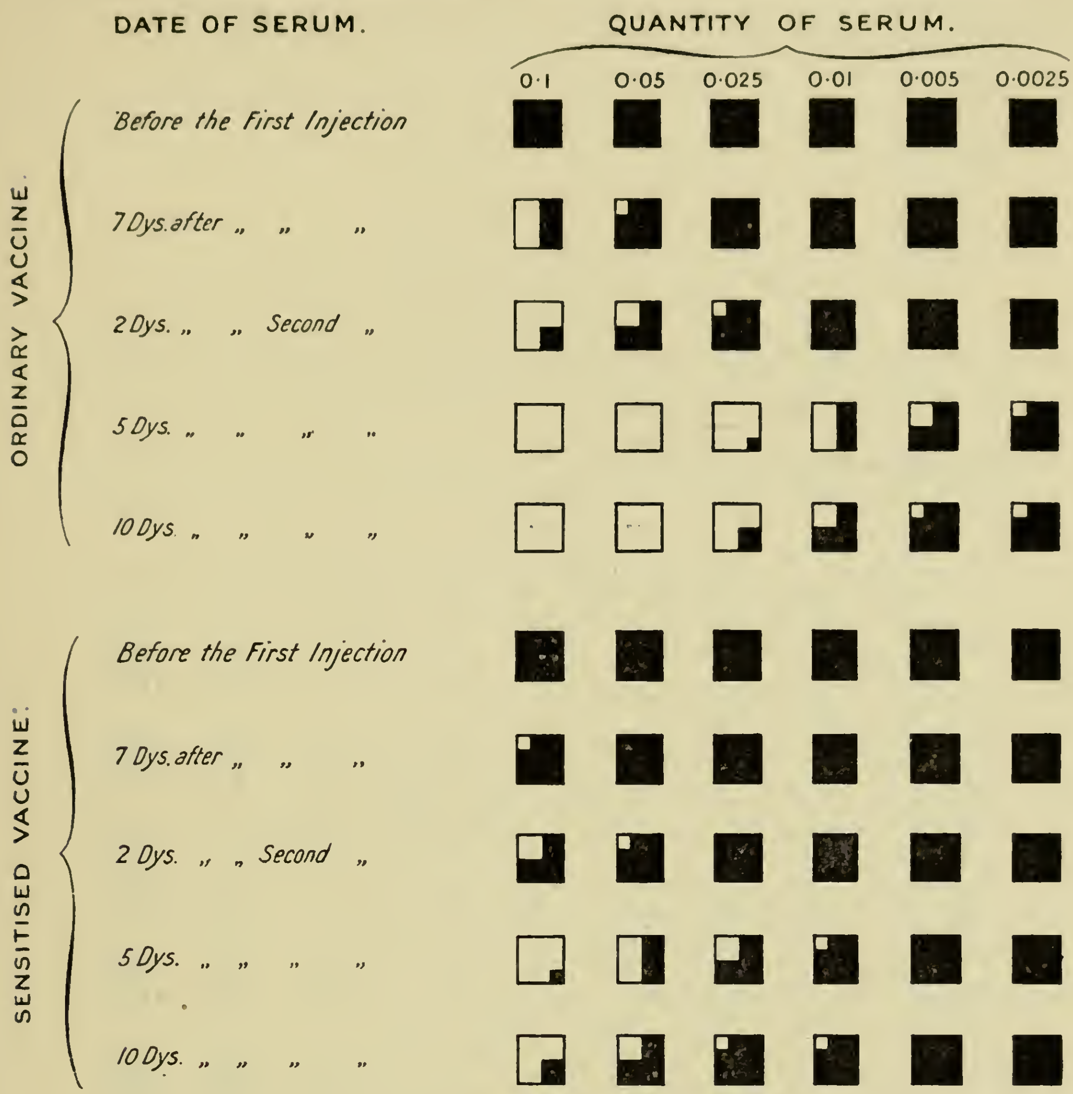
2. Complement-fixation.

The technique adopted was essentially that in common use. To 0·5 c.c. of each successive dilution of fresh serum heated at 56° C. for thirty minutes was added 0·5 c.c. of an emulsion of living bacilli containing approximately 1000 m. per cubic centimetre, and 0·5 c.c. of a dilution of fresh guinea-pig serum. This latter was so prepared as to contain in that quantity a minimal dose capable of completely dissolving within one hour the washed sheep-blood corpuscles in 0·5 c.c. of a 5 per cent. suspension in presence of the hæmolytic amboceptor. After one hour at 37° C., 0·5 c.c. of a 5 per cent. suspension of washed sheep-blood corpuscles was added to each tube, with a 0·5 c.c. of a dilution of hæmolytic amboceptor standardised with the same complement to contain three lytic doses. The mixture was then incubated two hours at 37° C., and a preliminary examination having been made, was left overnight in the cold, and then finally examined. The emulsion of bacilli used was a half-dose of the highest concentration, with which definite inhibition of hæmolysis did not occur, as shown by a previous test. The hæmolytic amboceptor was of course derived from rabbits injected with washed sheep-blood corpuscles. Further, a double dose of the serum adopted was found not to provoke any inhibition of hæmolysis. The other usual controls were always taken in the examination proper.

First, as in the case of agglutination, the mixtures of 5 serums obtained before and between the two injections, and at different intervals after, were examined as shown in Table XIX.

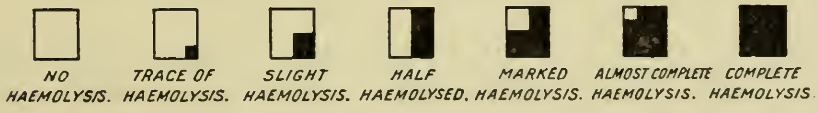
As in the case of agglutination, so also in the case of deviation of complement, the same relation is found to exist between the sensitised and non-sensitised vaccine, the degree of deviation being always far lower with the serum produced by the former, but with both varying directly with the time. Seven days after the first injection no clear difference is found; the sera of both series show little reaction. Two days after the second injection, however, the difference can be distinctly observed, and after five days, when the reaction is highest, it reaches the

TABLE XIX.—*Complement-fixation shown at Various Intervals by the Rabbits injected Twice.*


DATE OF SERUM.
QUANTITY OF SERUM.
0·1
0·05
0·025
0·01
0·005
0·0025
ORDINARY VACCINE.
Before the First Injection
7 Dys. after „ „ „
2 Dys. „ „ Second „
5 Dys. „ „ „ „
10 Dys. „ „ „ „
SENSITISED VACCINE.
Before the First Injection
7 Dys. after „ „ „
2 Dys. „ „ Second „
5 Dys. „ „ „ „
10 Dys. „ „ „ „

Complement in each tube 0·04, Haemolytic amboceptor 0·0015.

EXPLANATION OF SCHEME USED
TO ILLUSTRATE THE COMPLEMENT-FIXATION EXPERIMENTS.



Each tube in the original experiment is represented by a square in the Table.

maximum. Testing the degree of reaction at this time, we find that of the sensitised vaccine is one-fourth of that of the ordinary. So that the difference in this case is smaller than in the case of agglutination.

Further, this reaction was tested with two other kinds of injection, and the sera obtained seven days after the last injection were examined separately (see Tables XX. and XXI.).

Although there is great individual variety in the sera, with parallelism in agglutination, all those derived from the sensitised vaccine have a reaction lower than that of the weakest of those derived from the ordinary.

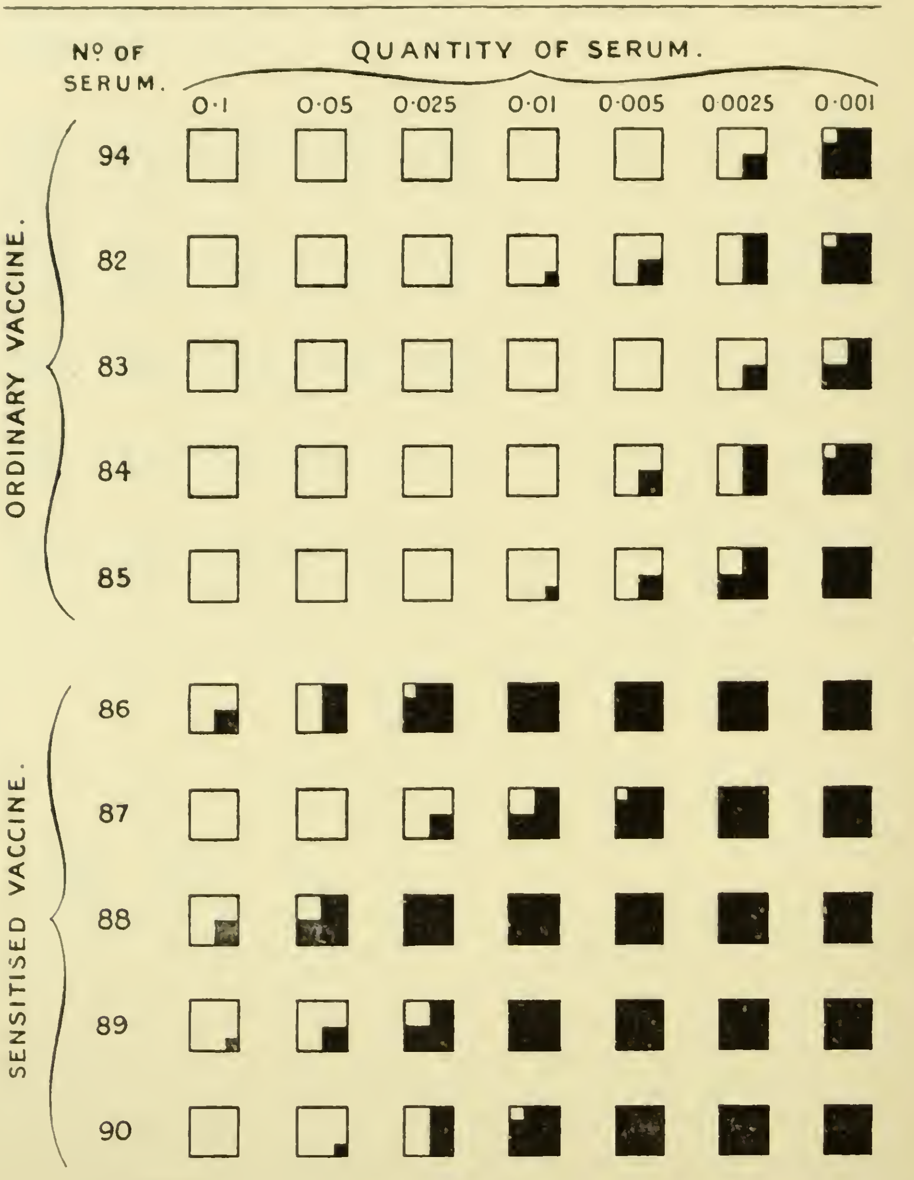
Thus we can see also with this organism that the sensitised killed vaccine produces fewer antibodies (agglutinin and complement deviation) than the ordinary, as Garbat and Meyer observed in the case of the typhoid bacillus.

IV. Comparison of the Degree of Immunity given by the Two Vaccines.

1. Determination of the Minimal Lethal Dose.

A 24-hour agar-slope culture of the *B. pseudo-tuberculosis rodentium* is suspended in some quantity of saline solution. After freeing from coarse bacillary clots in the centrifuge, a definite concentration is prepared

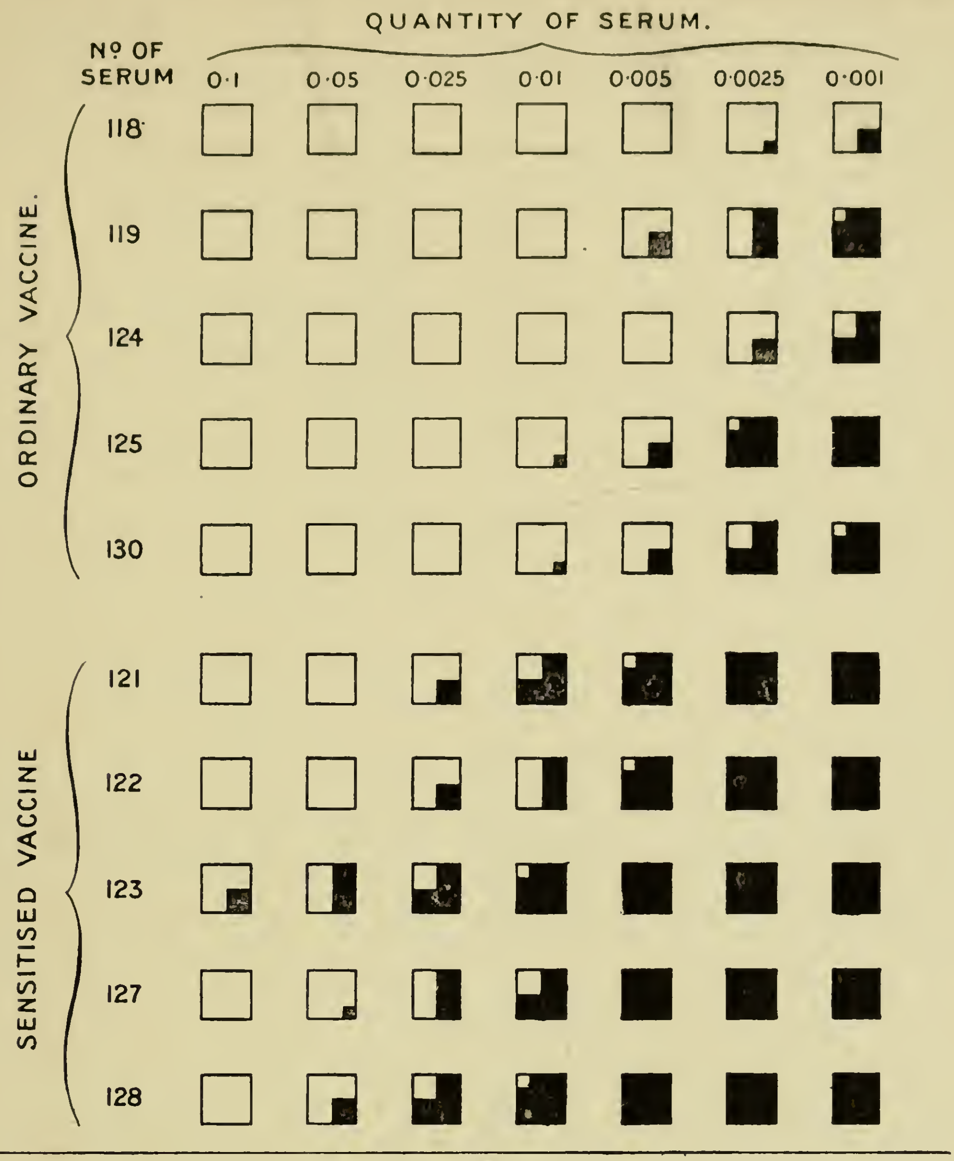
TABLE XX.—*Complement-fixation of the Sera produced by Three Injections.*



	Nº OF SERUM.	QUANTITY OF SERUM.						
		0·1	0·05	0·025	0·01	0·005	0·0025	0·001
ORDINARY VACCINE.	94	□	□	□	□	□	◲	◩
	82	□	□	□	◲	◲	◨	◩
	83	□	□	□	□	□	◲	◩
	84	□	□	□	□	◲	◨	◩
	85	□	□	□	◲	◲	◩	■
SENSITISED VACCINE.	86	◲	◨	◩	■	■	■	■
	87	□	□	◲	◩	◩	■	■
	88	◲	◩	■	■	■	■	■
	89	◲	◲	◩	■	■	■	■
	90	□	◲	◨	◩	■	■	■

Complement in each tube 0·03, Haemolytic amboceptor 0·003.

TABLE XXI.—*Complement-fixation of the Sera derived from Five Injections.*


QUANTITY OF SERUM.
Nº OF SERUM
0·1
0·05
0·025
0·01
0·005
0·0025
0·001
ORDINARY VACCINE.
118
119
124
125
130
SENSITISED VACCINE
121
122
123
127
128

Complement in each tube 0·03, Haemolytic amboceptor 0·003.

from this emulsion, as in the preparation of vaccine, in order that a number of cubic centimetres proportional to the number of kilograms in the rabbit's weight may be injected into the marginal ear vein. The rabbit thus injected is observed for some time, and after death a careful examination is made, together with bacteriological tests of the heart blood, gall, and peritoneal fluid (for results, see Table XXII.).

With the injection of 100 m. (at times 50 m.) of living bacilli per kg., though death occurs very quickly—within four to seven days—the post-mortem examination shows in most cases the features of acute intoxication, fatty liver, enlargement of spleen, acute nephritis, etc. After the injection of 20 m. per kg., however, death generally comes more slowly, within six to ten days, and always with the characteristic post-mortem appearances of pseudo-tuberculosis, with minute grey nodules in spleen and liver, and sometimes exudation in peritoneal cavity. But the injection of 10 m. per kg. also invariably kills the animals within nine to fourteen days, and the post-mortem gives us the same results as before. On the other hand, the injection of 5 m. per kg. was atal in only two cases out of six. Also in other cases, the animals actually

TABLE XXII.—*Determination of the Minimal Lethal Dose.*

Rabbit.			Injected Bacilli per Kg.	Days of Death after Infection.	Last Weight.	Post-mortem Appearance.	Isolation of Bacilli from Blood, Gall, and Peritoneal Fluid.
No.	Sex.	Weight.					
52	M.	3000	100 m.	7	2350	Characteristic.	+
76	M.	2280	100 ,,	4	2070	Not so marked.	+
77	F.	2200	100 ,,	5	1740	,,	+
16	M.	1850	50 ,,	9	1520	Characteristic.	+
22	F.	2375	50 ,,	5	2050	Not so marked.	+
105	F.	1460	50 ,,	7	1170	Characteristic.	+
168	F.	1960	20 ,,	10	1550	,,	+
203	M.	1820	20 ,,	6	1550	,,	+
204	F.	1540	20 ,,	7	1320	,,	+
207	F.	2040	20 ,,	7	1750	,,	+
175	F.	1630	10 ,,	9	1320	,,	+
201	M.	1860	10 ,,	10	1550	,,	+
202	M.	1540	10 ,,	14	1250	,,	+
210	F.	1860	10 ,,	10	1760	,,	+
34	F.	2040	5 ,,	...	...	Normal.	−
36	F.	1980	5 ,,	...	...	,,	−
218	M.	1860	5 ,,	...	...	,,	−
219	M.	1860	5 ,,	8	1540	Characteristic.	+
220	F.	1710	5 ,,	10	1230	,,	+
222	M.	1470	5 ,,	...	...	Normal.	−

remained in a state of high fever for some days after the injection, and then recovered sooner or later from the disease, gradually gaining in weight. When they were killed after a month and examined, no visible change could be found in the internal organs.

Thus the minimal lethal dose for the rabbit, given intravenously, was proved to be 10 m. per kg.

2. The Experiments proper.

The rabbits used for this experiment were those described in the preceding chapter, that is, the groups of rabbits immunised with successive injections of three kinds (twice, thrice, and five times at intervals of seven days). All the rabbits in each group were given the lethal dose simultaneously ten days after the last injection, and carefully observed for two to three weeks. Naturally, those that died of disease were always dissected, and, if necessary, bacteriological examination was made. Even the rabbits still surviving after a month, unless manifestly suffering great weakness, were killed to decide the existence of infection by post-mortem examination.

Experiment 1.—The twice injected rabbits were taken for this experiment. Ten days after the last injection, having been weighed, they were injected intravenously with lethal doses of two strengths per kg. (see Table XXIII.).

After injection of twice the minimal lethal dose, all the rabbits in both series died of infection. One rabbit (No. 166), however, immunised with the ordinary vaccine, remained alive for a fairly long time, but finally fell a victim to the disease, as the post-mortem examination definitely proved. On the other hand, after injection of the minimal lethal dose, two or three rabbits in each group bore the injection without any appreciable ill effects. After a month they were killed and proved quite healthy. Thus it is seen that, though the two kinds of vaccine differ greatly in their production of antibodies, yet in the power they give of resistance against infection, no pronounced difference is revealed.

TABLE XXIII.—*Comparative Resistance after immunising with Two Injections.*

Previous Treatment.		Rabbit.			Active Dilution of Serum for Agglutination.	Injection of Lethal Dose.		Cases of Death.		Infection Determined by Post-mortem Examination.
		No.	Sex.	Weight.		Date.	Quantity (per Kg.).	Days after Injection.	Last Weight.	
Immunised with Ordinary Vaccine.	1 .	158	F.	2200	640 +	21/vi	20 m.	6	1860	+
	2 .	159	M.	1860	640 ++	,,	,,	7	1500	+
	3 .	160	M.	1980	640 +	,,	,,	8	1680	+
	4 .	162	F.	2060	320 +	,,	,,	6	1800	+
	5 .	166	F.	2040	320 ++	,,	,,	36	1120	+
	6 .	151	M.	1300	640 ±	19/vii	10 m.	..	..	-
	7 .	184	F.	2080	320 +	,,	,,	..	..	-
	8 .	191	F.	1240	320 ±	,,	,,	7	1140	+
	9 .	192	F.	1340	320 ±	,,	,,	9	1120	+
	10 .	195	F.	1480	1280 +	,,	,,	..	..	-
Immunised with Sensitised Vaccine.	1 .	167	M.	2040	80 ±	21/vi	20 m.	7	1760	+
	2 .	171	F.	2340	80 +	,,	,,	11	1940	+
	3 .	173	F.	1980	80 ±	,,	,,	7	1630	+
	4 .	174	M.	1820	40 ++	,,	,,	5	1600	+
	5 .	176	M.	1880	40 +	,,	,,	8	1740	+
	6 .	156	F.	2120	40 +	19/vii	10 m.	..	..	-
	7 .	178	F.	1360	80 +	,,	,,	9	1120	+
	8 .	182	F.	1660	20 +	,,	,,	..	..	-
	9 .	185	F.	1880	40 +	,,	,,	17	1500	+
	10 .	193	F.	1460	40 ±	,,	,,	10	1060	+
Controls no Treatment.	1 .	205	M.	1820	..	21/vi	20 m.	7	1460	+
	2 .	216	M.	2160	..	,,	,,	7	1800	+
	3 .	198	F.	1720	..	19/vii	10 m.	9	1350	+
	4 .	200	F.	1440	..	,,	,,	10	1020	+
	5 .	209	F.	1730	..	,,	,,	14	900	+

Experiment 2.—The group of rabbits injected thrice with each vaccine was treated with twice and five times the minimal lethal dose ten days after the last injection, as in Experiment 1. The results in Table XXIV. show that the intensity of immunity is also about the same in both series.

After injection of 50 m. living bacilli per kg., no rabbit survived in either series of immunisation. But after injection of 20 m. per kg., three rabbits previously treated with the non-sensitised vaccine bore the injection safely, while two of those immunised with the sensitised were altogether free of infection.

Experiment 3.—Expecting that the gradual increase of injected doses in the immunisation would produce a much higher immunity in animals, the

TABLE XXIV.—*Comparative Resistance after immunising with Three Injections.*

Previous Treatment.		Rabbit.			Lowest Active Dilution of Serum for Agglutination.	Injection of Lethal Dose.		Cases of Death.		Infection Determined by Post-mortem Examination.
		No.	Sex.	Weight.		Date.	Quantity (per Kg.).	Days after Injection.	Last Weight.	
Immunised with Ordinary Vaccine.	1 .	82	F.	1620	640 +	26/iv	50 m.	4	1490	+
	2 .	83	F.	1970	2560 ±	,,	,,	17	1260	+
	3 .	84	F.	1800	1280 +	,,	,,	5	1630	+
	4 .	85	F.	1520	640 +	,,	,,	6	1350	+
	5 .	94	F.	1520	2560 ±	,,	,,	7	1380	+
	6 .	157	F.	2880	1280 +	28/vi	20 m.	..	..	–
	7 .	161	F.	2750	2660 ±	,,	,,	..	..	–
	8 .	163	F.	2780	1280 ±	,,	,,	..	..	–
	9 .	165	F.	2720	640 ±	,,	,,	10	2140	+
	10 .	177	F.	2240	640 +	,,	,,	9	2020	+
Immunised with Sensitised Vaccine.	1 .	86	F.	1490	80 +	26/iv	50 m.	15	1160	+
	2 .	87	F.	1370	320 ±	,,	,,	5	1240	+
	3 .	88	F.	1740	40 +	,,	,,	4	1450	+
	4 .	89	F.	1600	80 ±	,,	,,	5	1360	+
	5 .	90	M.	2070	160 +	,,	,,	6	1670	+
	6 .	169	M.	2250	160 ±	28/vi	20 m.	..	..	–
	7 .	170	F.	2180	160 +	,,	,,	7	1820	+
	8 .	172	F.	2720	80 +	,,	,,	..	..	–
	9 .	178	F.	2950	80 ±	,,	,,	6	2840	+
	10 .	180	F.	1780	320 ±	,,	,,	5	1340	+
Controls no Treatment.	1 .	113	M.	1850	..	26/iv	50 m.	6	1540	+
	2 .	114	F.	1540	..	,,	,,	5	1230	+
	3 .	221	F.	2050	..	28/vi	20 m.	6	1720	+
	4 .	223	M.	2700	..	,,	,,	7	2260	+
	5 .	208	F.	2000	..	,,	,,	7	1680	+

group of rabbits were injected five times, and ten days after the last injection given lethal doses of three strengths per kg., as shown in Table XXV.

In this case, also, the injection of 50 m. living bacilli per kg. could not be borne by any rabbit in either series. Yet one rabbit (No. 124) injected with the ordinary vaccine remained alive for a long time; finally, this one also died of infection. After the injection of lethal doses of 20 and 30 m. per kg., the number of rabbits that survived was about the same in both series.

Now, summarising the results obtained (Table XXVI.), we can conclude as follows: with regard to the degree of immunity against infection, namely, the intensity of bactericidal power *in vivo*, there is no appreciable difference between the two vaccines, as Rowland (1912 [39]) asserted. At least it is a certain fact that with the sensitised vaccine, animals do not obtain much higher

TABLE XXV.—*Comparative Resistance after immunising with Five Injections.*

Previous Treatment.	Rabbit.			Lowest Active Dilution of Serum for Agglutination.	Injection of Lethal Dose.		Cases of Death.		Infection Determined by Post-mortem Examination.
	No.	Sex.	Weight.		Date.	Quantity (per Kg.).	Days after Injection.	Last Weight.	
Immunised with Ordinary Vaccine. 1 .	118	M.	1130	5120 ±	14/vi	50 m.	7	1170	+
2 .	124	M.	1040	2560 ±	,,	,,	32	820	+
3 .	125	M.	1400	640 +	,,	,,	10	1120	+
4 .	132	F.	1560	1280 ±	,,	,,	5	1300	+
5 .	119	M.	1660	1280 ±	,,	30 m.	7	1600	+
6 .	130	F.	1760	1280 +	,,	,,	10	1590	+
7 .	136	F.	1600	1280 ±	,,	,,	9	1400	+
8 .	137	F.	1520	2560 +	,,	,,	..	..	–
9 .	150	M.	2260	2560 ±	22/vii	20 m.	..	..	–
10 .	183	F.	2580	1280 +	,,	,,	7	2250	+
11 .	187	M.	2180	1280 ±	,,	,,	..	..	–
12 .	188	M.	2580	1280 +	,,	,,	..	..	–
Immunised with Sensitised Vaccine. 1 .	122	F.	1320	320 +	14/vi	50 m.	6	1170	+
2 .	127	F.	1440	160 ±	,,	,,	10	1220	+
3 .	128	F.	1620	160 +	,,	,,	5	1280	+
4 .	138	F.	1250	40 ++	,,	,,	7	1110	+
5 .	121	F.	1640	320 ±	,,	30 m.	6	1460	+
6 .	123	F.	1440	40 ++	,,	,,	..	..	–
7 .	135	M.	1560	160 +	,,	,,	..	..	–
8 .	142	M.	2240	160 +	,,	,,	7	1330	+
9 .	186	F.	2840	160 +	22/vii	20 m.	..	..	–
10 .	189	F.	2500	640 +	,,	,,	..	..	–
11 .	190	F.	2600	80 ±	,,	,,	15	2000	+
12 .	197	M.	2720	80 ±	,,	,,	..	..	–
Controls no Treatment. 1 .	213	M.	1860	..	14/vi	50 m.	5	1800	+
2 .	214	M.	1700	..	,,	,,	6	1680	+
3 .	211	M.	1560	..	,,	30 m.	7	1420	+
4 .	212	M.	2060	..	,,	,,	7	1990	+
5 .	224	M.	1940	..	22/vii	20 m.	10	1480	+
6 .	225	M.	2080	..	,,	,,	8	1560	+

immunity than with the other. On the other hand, as agglutination and deviation of complement are much lower with the sensitised vaccine, we can see that in this case the amount of both the antibodies does not indicate the actual degree of immunity given by them. That is to say, this fact proves that the sensitising enables the killed bacilli injected to make, as Garbat and Meyer and others have shown, a partial increase of antibodies, but not of the same components as those suggested by Ardin-Delteil and his collaborators in case of the living bacilli.

TABLE XXVI.—*Summary of Results of injecting Immunised Rabbits with Various Doses.*

Doses per kg. –		50 m.		30 m.		20 m.		10 m.	
		No.	Not infected.	No.	Not infected.	No.	Not infected.	No.	Not infected.
Twice injected	Ordinary vaccine	...	...	...	...	5	0	5	3
	Sensitised ,,	...	...	...	...	5	0	5	2
Thrice injected	Ordinary vaccine	5	0	...	...	5	3	...	...
	Sensitised ,,	5	0	...	...	5	2	...	...
Five times injected	Ordinary vaccine	4	0	4	1	4	3	...	...
	Sensitised ,,	4	0	4	2	4	3	...	...

Thus the sensitising seems to me, apart from the fact that the process renders living bacilli less liable to produce carriers, as first reported by Metchnikoff and Besredka, to possess a slight advantage when applied to killed bacilli; as it renders the vaccine slightly less toxic without diminishing its immunising power, and this in spite of the comparative absence of agglutinin and complement-fixation.

Further, comparing the rabbits immunised with the same kind of vaccine in each series, especially with the sensitised vaccine, the degree of agglutination appears not to be quite proportional to that of resistance against the lethal dose. For instance, in Table XXIV. a rabbit (No. 180), the serum of which showed the highest agglutination of 1 : 320, died of infection very quickly, while another one (No. 172) survived, though with the lowest agglutination of 1 : 80. Such examples are found in each experiment with the sensitised vaccine, while in the case of non-sensitised they are not so marked. To explain such a mystery it must be remembered that the smaller rabbit is perhaps more sensitive to infection than the bigger, as was observed when immunising. Or, in accordance with the theory of Neisser and Lubowski, already mentioned, this may be due to the fact that the agglutination titre in the case of the sensitised vaccine appears so variable that it can hardly be utilised as an indication of the degree of immunity for the purposes of comparison.

SUMMARY.

Dealing with the *B. pseudo-tuberculosis rodentium* (Pfeiffer), the following are the effects upon the rabbit of the killed vaccine sensitised by Besredka's method, as compared with those of non-sensitised :—

1. The sensitised vaccine has one slight advantage in that the increase of temperature in injected animals is a little lower on the average than that produced by the non-sensitised, not only after the first injection, but also after succeeding injections.

2. The animals can generally bear the various series of injections of the sensitised vaccine with much less loss of weight than when the non-sensitised is used, so that the former appears to be less toxic, although the number of animals which died of injection is not sufficient to decide this point.

3. As to the degree of immunity obtained by the injection of each kind of vaccine, there is no appreciable difference, for they both give about the same degree of resistance against various lethal doses, when the animals are compared ten days after the last injection of vaccine.

4. The production of antibodies in the serum, testable by agglutination and complement-fixation, is much less with the sensitised than with the non-sensitised vaccine under the same conditions. Thus the estimation of these antibodies *in vitro* does not show the actual degree of immunity given by the former, as compared with the latter.

In conclusion, I must express my gratitude for valuable help and suggestions in conducting this research given in various ways by Prof. Martin of the Lister Institute, Dr. Ledingham, and Dr. Penfold.

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NOTE ON THE KEEPING QUALITIES OF THERAPEUTIC SERUMS.

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AT the present time, when it is necessary to keep large stocks of therapeutic serums on hand to meet any sudden call, it becomes of importance to know the rate at which serums deteriorate.

The serum which has been the most carefully studied in this respect is antidiphtherial serum.

Diphtheria Antitoxin.

Anderson (1910) examined the question under experimental conditions and found that the average yearly loss in unitage was about 20 per cent. when the serum was kept at room temperature (70° F.–95° F.); at 15° C. the loss was about 10 per cent., and at 5° C. about 6 per cent. per annum. Dry diphtheria antitoxin kept in the dark at 5° C. remains stable for at least five and a half years. Old serums are just as good as fresh serums when the relative unitage is taken into account.

The writer (1913) also carried out some experiments in this direction. His results confirm those of Anderson inasmuch as he found that the loss during one year amounted to 7 per cent. when the serum was kept in an ice chest, and to 14 per cent. when the store temperature varied between 6° C. in winter and 16° C. in summer. At 36° C. the loss was 37 per cent. in six months and 51 per cent. in twelve months. One serum kept in bulk (100 c.cm.), instead of in the ordinary ampoules, at 36° C. lost 62 per cent. of its antitoxin in six months.

We see from these observations what we can count upon when the serum is kept under proper conditions, namely, in the dark and in the cold.

But serums on the market are not always stored so carefully, judging from tests carried out on samples of serum which have been in commerce.

In the *Journal of the Royal Army Medical Corps*, vol. iii, 1904, p. 601, are given results of an examination of some samples of diphtheria antitoxin. The interval between the tests was about fourteen months, and during that time some of the samples had made two voyages to India and back. The maximum loss in unitage was 20 per cent., and the average loss some 7 to 8 per cent.

Miller (1905), who examined eighty-two samples of returned serum, mentions that "A half-dozen packages of serum had evidently been exposed to considerable heat, as the serum in them was firmly coagulated." He concluded that—

1. The high potency serums change more rapidly than the weaker, but in neither case is the loss of potency as rapid as is usually supposed.
2. The ordinary packages of serum retain their full therapeutic value for the time limit, which is often stamped on the labels.
3. The demand frequently made for fresh antitoxin is not justified.

Kinyoun and Hitchens (1907) tested 100 serums, samples of "returned antitoxin" which had been subject to the usual commercial conditions. The time between the first and final tests varied from thirteen to twenty-seven months. The loss in unitage varied from 0 to 48 per cent.; the latter occurred in eighteen months. Many of the serums were out for more than eighteen months without losing so much.

In the following table are given the results of an examination of a number of samples of serum which had been in commerce, and which were all returned at the same time. It must be noted that the tests were not carried closer than 50 units. Thus a 450 unit serum might be 475 units or more, a 300 unit serum might be 340, and so on. Serum No. 4, for instance, may originally have been say, 640, and the loss really 6 to 7 per cent., but as the serum was, for market purposes, originally reckoned as 600 units its loss in commerce was *nil*. These batches of serum were made up of bleedings which had been kept for varying periods before being mixed up to form a batch. Serum No. 7 was composed of serums which had been in store for from two to sixteen months before being mixed, and therefore the "age of serum" is put as two to sixteen months:

TABLE I.—*Showing the Loss of Antitoxin which occurred in Samples of Diphtheria Antitoxin which had been in Commerce for Various Periods.*

No. of Batch.	Age of Serum.*	Period between First and Final Tests.	Unitage when Sent Out.	Unitage when Returned.	Loss in Unitage.
	Months.	Months.			Per Cent.
1	19–24	72	500	400	20
2	6–23	55	450	300	33
3	11	46	900	800	11
4	4–14	51	600	600	*Nil.*
5	9–13	47	550	300	45
6	3–54	49	450	300	33
7	2–16	44	550	450	18
8	3–16	41	450	350	22
9	5–13	40	450	350	22
10	7	39	1,100	600	45
11	5–9	39	450	350	22
12	6–9	36	600	400	33
13	5–6	36	450	300	33
14	4–7	33	450	350	22
15	2–9	32	450	350	22
16	10–16	25	450	400	11
17	13–16	24	450	400	11
18	6–12	29	600	450	25
19	4–7	29	550	350	36
20	7–13	28	400	300	25
21	10–14	27	450	400	11
22	13–20	18	450	350	22
23	11–16	23	500	450	10
24	7–16	22	500	450	10
25	11–22	22	600	450	25
26	12–16	19	450	300	33
27	7–16	19	400	350	12
28	10	21	500	350	30
29	13–24	17	550	400	27
30	11–14	17	500	400	20
31	14–22	9	450	400	11

* That is, period between the date of collection and the date of first testing and sending out into commerce.

The average period during which these serums were on the market is about two and a half years, and the average loss in unitage during that period is about 23 per cent., or about 10 per cent. per annum. Nothing is known about the conditions under which these serums were stored while in commerce, but it is obvious that the rates of deterioration varied greatly. If we compare the losses in Nos. 19, 20, and 21, and also those in 26 and 27, we must conclude that unless we know how serum has been stored the putting on the label of a time expiry limit tells us nothing, and may lead to a false sense of security.

Tetanus Antitoxin.

Kept under similar conditions this antitoxin loses its strength at about the same rate as diphtheria antitoxin.

Antiplague Serum.

Taking the antitoxin content of this serum as a criterion, it has been found that antiplague serum when stored under proper conditions changes little in the course of two years. (Cf. MacConkey, 1912.)

Antidysentery Serum.

There is no generally accepted method of standardizing this serum. In these laboratories we have for several years used as test "toxin" the dry, powdered bodies of bacilli of the Shiga type. The strength of this powder has remained fairly constant for three years—$\frac{1}{20}$ mg. suspended in distilled water or salt solution and injected intravenously usually proving fatal to a rabbit of 1,000 to 1,500 grams in four to five days. The test dose, 1–5 mg., of this "toxin" is mixed with the serum and at once injected intravenously. Tested in this way, antidysentery serum appears to retain its "toxin" neutralizing power with little change for at least one and a half years. Some serum which was sent out to India, kept there in an ice chest for one year and then returned, showed, when retested, no deterioration.

Antiscorpion Serum.

This serum preserves its property of neutralizing scorpion venom with little change for two years.

Antimeningococcic Serum: Antistreptococcic Serum.

We cannot say how long these serums retain their value, as there is no method accepted as reliable for standardization purposes. A paper on the standardization of antimeningococcic serum has appeared in the *Journal of Immunology*, August, 1916, but this journal has not been available as yet.

General Remarks.

It is specially to be noted with regard to diphtheria antitoxin and tetanus antitoxin that the loss which occurs on keeping is in the number of units only. The *quality* of the antitoxin remains the same, but the *quantity* is less. This decrease can easily be remedied by giving larger doses.

It would add greatly to our knowledge if, when either antiplague serum or antidysentery serum is used, a note were made of the distinguishing mark on the bottle. If this were always done and mentioned in reports we should in time possibly be able to establish a relationship between the quality of the serum and the therapeutic effect.

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No 11

ON

THE PASSIVE IMMUNITY CONFERRED BY A PROPHYLACTIC DOSE OF ANTITETANIC SERUM

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ON THE

PASSIVE IMMUNITY CONFERRED BY A PROPHYLACTIC DOSE OF ANTI-TETANIC SERUM.

THE occurrence of cases of tetanus even though a prophylactic injection of serum has been given draws attention to the comparatively short duration of the complete passive immunity conferred by a dose of antitoxin. There are many references to this in the literature from 1893 (Roux and Vaillard) down to the present time (Vaillard, 1916), and yet this fact does not seem to be as fully recognised as it ought to be even now.

It has been stated that after a subcutaneous injection of antitoxin the antitoxin content of the blood reaches its maximum in 2–3 days and then remains fairly constant till about the seventh day, when it begins to decrease. It is also said that a sufficient dose of antitoxin gives complete protection for several days and partial protection for 2–3 weeks. The object of the following communication is to record some experiments which bear on this point. These experiments form part of a larger series which was undertaken with the object of ascertaining the most economical, and at the same time reliable, method of using tetanus antitoxin.

Our first object was to find out the smallest quantity of antitoxin which would give complete protection for one week. For this purpose several series of guinea-pigs were taken and each animal in a series received a subcutaneous injection of the same amount of antitoxin, but the number of units given to each series was different. Seven days later the immunised animals and a series of controls received an injection of tetanus toxin subcutaneously. The experiment was repeated until the results were quite clear, as may be seen in Table I.

Note.—To avoid repetition it may be stated that all injections were given subcutaneously in the abdominal region.

TABLE I.

Dose of toxin (U.S.A.).	Anti-toxin units.			Controls	Anti-toxin units.			Controls.	Anti-toxin units.			Controls.	Anti-toxin units.			Controls.
	3½	7	10½		3½	7	10½		1	2	3		¼	½	¾	
0 000002 g	N	N	N	Sl	...			..	..	...	.	..	...	..	...	...
0·000003 g	,,	.	,,	Mo	N	N	N	Tet.*	N	N	N	+5	N	N	Sl	...
0·000004 g	..	,,	,,	..	,,	..	,,	+4	,	..	..	+5	Sl	Sl	Mo	...
0·000005 g	..	..	..	Ml	,,	,,	..	+4	,,	..	..	+3	+12	..	N	+6
0·0 0006 g	,,	..	,,	,	,,	,,	..	+4	.	..	.	+3	Ml	N	Tr	...
0·000007 g	..	.	.	...	,,	,,	,,	+3	..	,	..	+3	,,	Sl	,,	+4-5

* Tetanus, but recovered. N = Nil (no symptoms of tetanus). Sl = Slight attack of tetanus. Ml = Mild attack. Mo = Moderate attack. Tr = trace. + = Death, and the number which follows indicates the day on which death occurred. Two numbers (4-5) refer to the night between two days.

We see that ¼, ½, or ¾ U.S.A. unit does not afford complete protection for one week, though these amounts saved life against a dose of toxin which was fatal to the controls. If, however, we give 1 unit or more we obtain complete immunity during at least one week. As an ordinary man is about 220 times heavier than these guinea-pigs, this would mean that, calculating by body-weight only, a man would require some 250 U.S.A. units to protect him for a week, while a dose of 500 U.S.A. units would allow for man being twice as susceptible as the guinea-pig.

It might be urged that the conditions of the experiment were not similar to those occurring in actual practice. In order to meet this objection an experiment was carried out in which each animal received a small dose of toxin some time before the serum and then further small doses of toxin at short intervals, until by the seventh day each animal had received a lethal dose of toxin (0·000006 g.). The results are shown in Table II.

TABLE II.

No. of animal.	Days—							Result.
	1	2		3	4	6	7	
1-5	0·000001g toxin	3 units of antitoxin		...	0·000001g toxin	...	0·000004g toxin	N
		10 A.M.	3 P.M.					
6-10	0·000001g toxin	6 units	0·000001g toxin	0·000001g toxin	0·000001g toxin	0·000002g toxin	...	N
11-15	0·000001g toxin	9 units	0·000001g toxin	0·000001g toxin	0·000001g toxin	0·000002g toxin	...	N

N = Nil (no symptoms of tetanus).

It is obvious that giving part of the toxin (1/6 of a lethal dose) before the serum has made no difference to the result.

If we increase the interval between antitoxin and toxin from 1 week to 10 days we find that the immunity is beginning to diminish, and if we wait 2 weeks before testing the immunity we obtain results which confirm those of previous observers and which show that most of the protection afforded by an injection of antitoxin has disappeared. There may, however, be a partial immunity sufficient to save life, though not to prevent an attack of tetanus. (Table III.)

TABLE III.—*Immunity after 10 Days.*

Dose of toxin.	Dose of antitoxin in units.				Controls.
	1	3	6	9	
0·000004 g	Trace for 4 days.	Nil.	Nil.	Nil.	Mild tet., recd.
0·000005 g	Nil.	S.L.T. for 3 days.	,,	,,	+5-6
0·000006 g	,,	Nil.	+ 7-8	,,	...
0·000007 g	S.L.T. for 1 week.	Mild tetanus.	S.L.T. for about 1 week.	S.L.T. for about 1 week.	+4-5

S.L.T. = slight local tetanus.

TABLE IV.—*Immunity after 2 Weeks.*

Dose of toxin.	Antitoxin units.			Controls.	Antitoxin units.			Controls.
	3½	7	10½		3	6	9	
0·000003 g	Trace.	Slight.	Mild.	Slight.	...	..	...	K 13*
0·000004 g	+7	+8	+6	+6–7	Trace	+8–9	Mod.	...
0·000005 g	+7	Trace.	Slight.	+6–7	.	+7	,,	+6
0·000006 g	Trace	,,	+6	+6–7	K 5*	+6	Slight.	..
0·000007 g	Mild.	,,	+7	+6	Mod.	Trace	,,	+4–5

* K 5 and K 13 = killed on fifth and thirteenth day respectively because of severity of the attack.

When the immunity has diminished so much in the course of a fortnight after an injection of tetanus antitoxin one is not surprised to find that practically all is gone at the end of three weeks.

TABLE V.—*Immunity after 3 Weeks.*

Dose of toxin.	Number of units of antitoxin.				Controls.
	1	3	6	9	
0·000004 g	+ 5–6	+ 7–8	Mod. tet. recd.	+ 8–9	Mod. tet. recd.
0·000005 g	+ 4–5	+ 4	+ 4–5	Mod. tet. recd.	+ 8–9
0·000006 g	+11–12	+ 4–5	+ 5	+ 5–6	..
0·000007 g	+ 4	+ 3–4	+ 4–5	+ 5–6	+ 4–5

From these results it would seem that the increased dose of antitoxin has not had an appreciable effect in increasing the duration of the subsequent immunity.

In view of these results it seemed advisable to try whether a very much larger dose than had so far been given would give a correspondingly better result. Each of 5 guinea-pigs received beneath the skin of the abdomen 40 U.S.A. units of tetanus antitoxin, and after an interval of 2 weeks the test doses of toxin. (Table VI.)

TABLE VI.—*Immunity 2 Weeks after 40 U.S.A. Units of Antitoxin.*

Antitoxin.	Toxin.	Result.
40 U.S.A. units.	0·000003 g	No symptoms of tetanus.
,,	0·000004 g	Mild tetanus from 5th to about 15th day
,,	0·000005 g	Trace of local tetanus 8th–15th day.
,,	0·000006 g	,, ,, ,,
,,	0·000007 g	Mild tetanus from 7th to about 20th day

We see, then, that even such a large dose as 40 U.S.A. units (corresponding to at least 8000 units in man) does not afford complete protection for a fortnight. It would also seem that Levin (1909) was right when he expressed the opinion that we cannot hope by increasing the dose of antitoxin to increase greatly the duration of passive immunity.

These results led us to go a step further and to give an enormous dose, a dose which would allow us to compare our results with those of Ruediger (1913), who stated that 250 U.S.A. units of tetanus antitoxin would protect guinea-pigs for from four to six weeks against a dose fatal to the controls. Unfortunately, he does not mention whether the protection was complete or not. We gave to each of a series of guinea-pigs 250 U.S.A. units of antitoxin and tested their immunity at the end of four weeks. (Table VII.)

TABLE VII.—*Immunity 4 Weeks After 250 Units of Antitoxin.*

Antitoxin.	Toxin.	Result.
250 U.S.A. units.	0·000006 g	Mild tetanus—well within 3 weeks.
,,	,,	Moderate ,, — ,, ,,
,,	,,	,, ,, — ,, ,, ,,
,,	,,	Slight symptoms.
,,	,,	Mild attack—well within 3 weeks.

This large dose has protected, but not completely, though there was never any question of life being endangered. Now there happened to be available 13 guinea-pigs which had each received 10 c.c. of antitetanic serum in the ordinary course of routine testing. To each of these animals was given beneath the skin of the abdomen the official minimal lethal dose of the standard dry powdered test toxin which has been used in all the (previous) tests detailed in the paper. (Table VIII.)

TABLE VIII.

No.	Anti-toxin.	Interval between injection of antitoxin and of toxin	Toxin.	Result.
	U.S.A. units.	Days.		
1	9000	27	0·00000·6 g	No symptoms of tetanus.
2	3000	27	,,	,, ,,
3	3000	27	,,	Trace of tetanus present from 3rd–6th day and then passed off.
4	2200	31	,,	Very slight symptoms; recovery.
5	2200	31	,,	,, ,,
6	3000	40	,,	Very slight tetanus. Death 21st day.
7	3000	40	,,	Tetanus. Death 6th day.
8	3500	47	,,	,, ,,
9	3500	47	,,	Slight tetanus; recovery.
10	3500	40	,,	,, ,,
11	3500	40	,,	Mild tetanus; recovery.
12	3000	28	,,	Only a trace; recovery.
13	3000	28	,,	No symptoms.

A consideration of the results of these experiments fixes our attention on two points which stand out prominently. The one is the extreme differences in the susceptibility of guinea-pigs to tetanus toxin; the other is the enormous doses which apparently we must give when we aim at obtaining passive immunity of long duration. Of course, it would, in present circumstances, be quite impracticable to use for man doses corresponding to some of those we have given to guinea-pigs, and we must content ourselves with the smaller doses, even though the protection does not last as long as we would wish.

We have now to decide upon what is the best thing to do, knowing that we cannot count upon producing an immunity of long duration by the use of a single prophylactic dose of tetanus antitoxin. There are two ways of dealing with this question. On the one hand, we might, after giving the prophylactic dose, simply keep very careful watch for the first signs of incipient tetanus and then at once treat the case energetically. We have not noticed in the literature any suggestion that this course should be followed, though it is possible that as our knowledge of the disease and its early symptoms increases, it may turn out to be after all the best practice. On the other hand, we may repeat the protective dose with the object of keeping up the immunity. There is in favour of this latter view a large volume of opinion, much of it recent and based on experience gained since the war began.

Park and Williams (1914, p. 331) say that where tetanus infection is suspected the antitoxic serum should be given a second, or even third, time at intervals of seven days. V. Behring (1914) considers that if the infection of the wound continues the prophylactic dose should be repeated and antitoxin might also be injected locally.

Teutschländer (1915) goes so far as to recommend that as long as there may be tetanus bacilli in the body, then if thorough surgical treatment with removal of all foreign bodies—including their capsules when encapsulated—cannot be carried out, a *curative* dose of serum should be given whenever the temperature rises or there is any suspicious change in the condition.

Bazy (1916) is in favour of repeating the injections every eight days for a month. Among others who express opinions similar to the above may be mentioned Dubs (1915), Witzel (1915), Fraenkel (1915), Aschoff (1915), H. E. Robertson (1916), Kümmell (1916), Bérard and Lumière (1916), Montais (1916), and Capitan (1916).

Finally, we may quote from a communication made by M. Vaillard to the Academy of Medicine, Paris, on Sept. 5th, 1916, in which he says:—

"...... le Sous-Secrétaire d'Etat au Service de Santé a bien voulu me confier, de concert avec notre collègue

M. Roux, l'examen des observations recueillies, pour en déduire les enseignements qu'elles comportent sur la prevention du tétanos. Les considérations présentées à la suite de cet examen lui ont inspiré une instruction en date 10 Juin, 1916 ; j'ai cru devoir les communiquer à l'Académie."

He mentions that the passive immunity conferred by a dose of antitoxin lasts only about 8–12 days, and that if toxin is still being produced in the body after this lapse of time there will be no antitoxin present to neutralise it, and then :—

"Si au contraire la provision d'antitoxine est renouvelée en temps opportun, la preservation sera prorogée pour un nouveau laps de temps après chaque nouvelle injection. De là, pour certaines blessures particulièrement dangereuses, l'absolue nécessité de ne pas confier l'immunisation à une seule injection préventive, et de réitérer ces injections tant que persiste la menace du foyer infecté d'où peut sortir le tétanos."

He then goes on to speak of late tetanus, and concludes :—

"De ces considérations on est conduit à déduire que les *réinjections* méthodiques de serum antitoxique fourniront un des moyens de mieux assurer la prevention du tétanos consecutif aux blessures récentes et d'étendre le même bénéfice aux periodes plus ou moins éloignées de l'evolution des plaies. Il serait regrettable que la crainte de l'anaphylaxie dont la survenance a été exagérée surtout à la suite des injections sous-cutanées détournât les chirurgiens d'une pratique judicieuse qui a fait ses preuves."

This question of anaphylaxis was brought prominently forward by M. de Massary before the Société Médicale des Hôpitaux de Paris in October, 1915, when he referred to the case of a soldier who was wounded and received an injection of serum. Wounded again a month later, he told the surgeon of the previous injection. Owing apparently to fear of anaphylaxis a second dose of antitoxin was not given. Five days later tetanus supervened and ended fatally.

The discussion which followed this communication ended in the appointment of a committee to inquire into the question of the effect of reinjections of serum. The committee was composed of MM. Achard, Aviragnat, Barbier, Chantemesse, Marfan, Netter, Siredey, and Louis Martin, who, after very careful consideration of the subject, drew up a report which was published in the *Bull. Société Méd. des*

Hopitaux de Paris, Nov. 19th, 1915, p. 1076, and which may be summarised as follows:—

They define a "reinjection" of serum as any injection which is made not earlier than 15 days after the previous injection.

Intravenous reinjections, they consider, should not be used as a routine measure, but be limited to cases of plague and grave tetanus, and above everything the injection should be made very slowly so that it can be stopped if necessary before much has been given.

Of *intraspinal reinjections* they say that it is impossible to give indications or contra-indications, for it is a surprising fact that in the same patient these injections are sometimes well borne and sometimes give rise to anxiety. The serum should be run in very, very slowly, and the needle left *in situ* for a time so that, should any disquieting symptoms occur, as much fluid as is necessary may be allowed to flow out again. When all fear of trouble is over the needle may be removed.

Subcutaneous reinjections are rarely followed by grave symptoms, and the risk of their occurrence cannot be compared with the risks run by the patient if a reinjection is not made. When there is urgent need for it the reinjection of serum should never be omitted owing to the fear of anaphylactic trouble.

Reinjections should not be given after a meal during the period of digestion, and should be given where the cellular tissue is very lax. They should be given slowly and stopped at the first sign of trouble. One should be doubly careful in the case of tuberculous subjects, asthmatics, those subject to urticaria, hay fever, or who are affected by various scents (e.g., smell of horses), or where there is any suspicion of purpura. In such conditions one should proceed very slowly with the first portions of the injection, one should stop at the slightest sign of anaphylaxis, and then, if all is well, begin again an hour or so later.

The final conclusion of the committee is that:—"On ne peut, dans aucun cas, reprocher à un médecin d'avoir pratiqué une réinjection de serum dans un but prophylactique ou thêrapeutique."

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CONCERNING THE PATHOLOGY AND ETIOLOGY OF THE INFECTIOUS JAUNDICE COMMON AT THE DARDANELLES, 1915.

BY

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DURING the autumn of 1915 troops at Gallipoli, and to a lesser extent in Egypt, suffered from a nearly non-fatal form of infectious jaundice, usually without complete obstruction to the entry of bile into the intestine, and not associated with any tendency to haemorrhages. It appeared to be markedly infectious; some units had 25 per cent. of their strength affected, and many cases occurred amongst patients in hospital for other complaints.

The first case occurred in the French lines about August 10th; during September the epidemic spread slowly until the end of that month, when it rapidly extended, reaching its maximum in October. During November it rapidly declined, and was practically over by the end of the year.

The following is an account of a case of moderate severity. All variations between this and mild jaundice without any obvious signs of illness were common.

The onset is characterized by lassitude, loss of appetite, headache, and often suffusion of conjunctivae. It is ushered in with a brief period of pyrexia, temperature 101–102°, and generally some pain in the upper abdomen. The liver becomes slightly enlarged, can be felt below the ribs, and is tender to the touch. The spleen is sometimes enlarged, but not tender.

Although the temperature may have returned to normal, these symptoms continue for three to five days, when jaundice occurs and lasts one to two weeks, or longer. The urine is bile-stained and the pulse often slow whilst jaundice is present.

By the time that jaundice is developed the patients generally feel better, but weakness continues, with rapid pulse and breathlessness on the least exertion.

Seven to ten days after the onset of the disease a distinct increase in the area of cardiac dullness may be made out. Slight albuminuria is not uncommon.

Convalescence is slow and the patient is not good for much for a month or longer.

According to the accounts of the disease published by Sarraillhé and Clunet (1916), Willcox (1916), and by Hurst (1916), the gall bladder is often, or generally, palpable and tender. Specific enlargement of the gall bladder was not discoverable in the cases I saw, nor was this the case in the experience of my colleagues. Sarraillhé and Clunet also record two cases of "ictère grave" which developed in forty-eight hours with high temperature, speedy collapse, purpura, haemorrhages, and death. They do not mention the existence of nephritis in these.

In opening the discussion on cases of jaundice at Alexandria in November, 1915, Professor Kartulis gave a brief survey of the varieties met with in Egypt and the Levant. He described two types of epidemic jaundice as not uncommon. The first is a severe disease of sudden onset with rigor, high temperature, intense headache, vomiting, and prostration. The liver becomes enlarged and tender, and the urine contains albumin and casts. On the fourth or fifth day jaundice appears. Epistaxis, haematuria, and petechial haemorrhages are characteristic features. The urine diminishes in amount and the patient becomes unconscious or delirious. Death occurs in 30 per cent. or upwards of the cases from uraemia, haemorrhages, or secondary infections. Convalescence is very prolonged.

The milder type described by Professor Kartulis resembles the disease encountered at Gallipoli as described above, but in the Egyptian cases nephritis, epistaxis, and petechial haemorrhages would appear to be usual symptoms.

Professor Kartulis had by means of various microscopical and bacteriological methods searched for the pathogenic agent in both classes of infectious jaundice, but hitherto in vain. He suggests that it must be invisible, like that of yellow fever.

The severe form resembles very closely the infectious jaundice prevalent in certain coal mines in Japan which, towards the end of 1914, was shown by Inada, Ido, Hoki, Kaneko, and Ito (1916) to be caused by a spirochaete. They accordingly named the microbe *Spirochaeta icterohaemorrhagiae*, and the disease spirochaetosis icterohaemorrhagica. Later Ido, Hoki, Ito, and Wani (1916) found the spirochaete in 39 per cent. of the rats in the mines, and they suggest that the miners became infected from these rodents.

Spirochaetosis icterohaemorrhagica has been also described by Hübener and Reiter (1916), Gwyn and Ower

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* This was first pointed out by Lieutenant-Colonel Willcox.

(1916), Stokes and Ryle (1916), and Martin and Pettit (1916), as occurring in the German, British, and French trenches.

In the spirochaetal disease, in addition to hepatitis and jaundice, haemorrhages and an acute nephritis occur. It has a mortality of 20 to 30 per cent., and is apparently dependent upon man's association with rats for its propagation. As described above, the Gallipoli disease was much milder, was apparently more infectious, and there was no reason to suppose that rats contributed to its dissemination. Nevertheless certain striking analogies occur, and the milder types of cases described by Kartulis show many characters intermediate between our comparatively mild disease and spirochaetosis icterohaemorrhagica.

SEARCH FOR THE CAUSE OF THE DISEASE.

At Lemnos in the autumn of 1915 I was not in a position to make animal experiments. I hunted for parasites in the blood of early cases, using dark-ground illumination with fresh films and also preparations stained with Giemsa's stain, but without success. I also made blood cultures in eighteen cases, but the only positive result was that I once isolated *B. typhosus*. This patient subsequently went through an ordinary attack of typhoid fever.

The experience of Captain Campbell (1916) at Cape Helles was similar. He made sixty-four blood cultures in non-febrile cases of jaundice and obtained negative results. On the other hand, from nine blood cultures of cases running a continuous temperature he recovered paratyphoid bacilli. In five instances the organism was *B. paratyphosus* B.

Sarrailhé and Clunet had, I understood, found an organism resembling *B. paratyphosus* in the blood in a number of cases of jaundice at Cape Helles, and were of opinion that the jaundice was merely a manifestation of paratyphoid fever. I therefore tested the serums of 37 cases two or three weeks subsequent to the onset of an attack of jaundice. Fifteen of the cases had had continuous fever for some time. Out of the 15 the serums of 7 agglutinated *B. paratyphosus* A and 2 *B. paratyphosus* B. The serums of the remaining 22 cases which had had no continuous fever gave no agglutination with either of the paratyphoid bacilli in a dilution of 1 in 50.

As both jaundice and paratyphoid fevers, especially paratyphoid A, were very common at the Dardanelles during October and November, 1915, it is not unreasonable to suppose that in some instances the two diseases were superimposed. As previously mentioned, a number of patients contracted jaundice in the hospital. In some cases the jaundice may have supervened as a complication of paratyphoid, cholecystitis being a not uncommon sequela of this disease. Jaundice, however, is rare.

Another line of inquiry was undertaken at Major Hurst's suggestion, and with his help. Having with him an

Einhorn's evacuator, Major Hurst persuaded patients to swallow it, and, when it had passed on into the duodenum, aspirated a few cubic centimetres of the duodenal contents, which were submitted to bacteriological examination forthwith. Duodenal samples from 8 cases of jaundice and from 4 normal individuals, including Major Hurst and myself, were examined; 0.02 c.cm. of the material was plated out upon MacConkey's bile salt agar, and also upon ordinary nutrient agar. Specimens of the different types of colonies were picked off, sown upon agar slopes, and subsequently investigated. The investigation of the cultures was carried out by my colleague, Sister F. S. Williams, for which, and for much other help with the observations in this inquiry, I am deeply grateful. The results were as follows:

> Three of the plates were sterile—two jaundice and one control.
>
> Nine of the plates grew organisms varying from 10 to 300 per plate.
>
> Five types of bacilli were isolated.
>
> Four types of cocci were isolated.

Only one type of microbe was universally present, and accounted for the great majority of the colonies. This was a small, non-motile, Gram-negative bacillus, which fermented no sugar, and did not form indol. This bacillus was present in all six of the plates from jaundice cases which grew anything at all, and three of the controls. It is apparently a non-motile member of the *faecalis alcaligenes* group common in the upper part of the alimentary canal. On first culture some of these colonies were much denser than others, but on subculture this difference tended to disappear.

The remaining types of bacilli were also all Gram-negative. Their characteristics are given below. Two of these types occurred in Case v, one in Case ii, and one in Case viii.

TYPES OF MICROBE CULTIVATED FROM DUODENUM.

Bacilli.

Appearance of Colony.	Appearance of Bacillus.	Fermentation of Sugars.					Indol.
		Lactose.	Glucose.	Mannite.	Dulcite.	Saccharose.	
A. Translucent	Short non-motile	A	A	A	0	0	0
B. Translucent	Short motile	0	0	A	0	0	0
C. Dense white	Short motile	A G	A G	A G	0	A G	0
D. Dense white	Short motile	0	A	A	0	0	0

Cocci.

The cocci were all Gram-positive with a tendency to form diplococci; two types occurred in Case IV, one in Case II, and one in Case V.

Appearance of Colony.	Appearance of Microbe.	Fermentation of Sugars.					Indol.
		Lactose.	Glucose.	Mannite.	Dulcite.	Saccharose.	
A. Dense white ...	—	0	0	0	0	0	0
B. Small glistening	—	0	A	0	0	0	0
C. White	—	0	A	0	0	A	0
D. Large yellowish	—	A	A	A	0	A	0

The only organism showing any general association with jaundice was the bacillus of the *faecalis alcaligenes* group and that was equally abundant in the controls. The other microbes were distributed haphazard, so that the results lend no support to the view that the jaundice is due to a microbial infection of the duodenum extending up the bile ducts.

MORBID ANATOMY.

As mentioned above, jaundice at Gallipoli was rarely fatal. Three cases died in No. 3 Australian General Hospital, in whom jaundice was still present at the time of death. In two of these the cause of death was cardiac syncope occurring some weeks after the onset of the illness, in the other jaundice supervened during an attack of paratyphoid B fever. *Post-mortem* examinations were made upon these cases by my colleague Major Upjohn, but as in two patients death occurred during convalescence, and in the case of the third the jaundice might have been a complication of his paratyphoid fever, one cannot expect to derive therefrom much information as to the essential pathology of the disease.

The following is a brief epitome of the notes made by Major Upjohn at the autopsies.

CASE I.

A young Maori admitted October 3rd, 1915, with fever, diarrhoea, abdominal pain, and vomiting. Symptoms maintained until October 10th, 1915, when he became delirious and violent, and died on October 11th. Slight jaundice developed a few days before death.

Post mortem: Heart and lungs normal; spleen enlarged. Liver enlarged, yellow, tough; excess of fibrous stroma. Gall bladder full of bile, which could, however, be slowly emptied into the duodenum by slight pressure. Small intestine injected, especially Peyer's patches in lower portion, no ulceration. Brain normal. *B. paratyphosus* B was recovered from the spleen and gall bladder.

CASE II.

Pte. C., aged 41, admitted October 25th, 1915, with slight rise of temperature, pain and tenderness over liver, jaundice, and great lassitude. On November 7th he was convalescing slowly, still weak and breathless. On November 21st he died suddenly during slight exertion.

Post mortem: Mildly jaundiced. Heart dilated, flabby, muscle light in colour. Liver usual size and consistency; olive brown in colour, gall bladder empty. Spleen normal size and appearance. Intestines normal. Spleen and bile sterile.

CASE III.

Pte. McP., aged 35, suffered from jaundice some weeks previously. Admitted from a convalescent dépôt, where he had been taken suddenly ill with rapid pulse and breathlessness, and pain in right hypochondrium. He died four hours after admission.

Post mortem: Heart dilated and flabby, muscle pale. Liver large, greenish, friable; gall bladder contained a small amount of bile. Spleen enlarged, soft. Kidneys pale, enlarged, and friable. Bile and spleen sterile.

Morbid Histology of the Liver.

The histological changes in the liver of both the cases which died some weeks after the onset of the jaundice attack were similar. Sections stained with logwood and viewed with the naked eye or a hand lens presented a marbled appearance, the outer portion of the lobules being stained, the inner colourless. Small deeply-staining areas where compensatory hypertrophy of liver tissue had occurred were scattered throughout the sections.

In each portal area there was small celled infiltration, with young fibrous tissue, and large cells full of pigment granules. The epithelium cells of the small bile ducts were proliferated, and many of the ducts appeared not to be patent. Deposits of the same pigment granules existed between the cells.

The outer half of the liver cells of the lobule appeared normal, but the inner half were more or less damaged. The cells adjacent to the intralobular veins were necrosed and indistinguishable as liver cells; more peripherally they were vacuolated. They did not contain fat. Amongst the degenerated cells were a number containing pigment granules. The distribution of this pathological change was not uniform, some groups of lobules being more severely affected than others. There was no dilatation of the blood capillaries. No bacteria were seen.

Small portions of the liver of both patients were treated by Levaditi's silver method for demonstrating spirochaetes. In neither case was there any suggestion of their presence.

The gist of the above results was communicated at the discussion on jaundice at the Anzac Medical Association, Cairo, on February 6th, 1916. At that time it did not seem worth while putting them on record, as I expected to encounter the disease again in Egypt the next autumn, and to be able to carry out experiments on animals. The disease did not, however, reappear among troops in epidemic form during 1916, so that I had no chance of completing the inquiry.

My object in recording them at this juncture is two-fold. First, the information may direct future investigators along more fruitful lines of inquiry; and secondly, because in the meantime conclusions regarding the etiology and pathology of the disease have been published which seem to be open to question.

MM. Sarraillhé and Clunet (1916), the pathologists to the French force at Cape Helles, conclude that the epidemic jaundice at Gallipoli was merely a manifestation of paratyphoid A fever. These gentlemen were in the habit of making blood cultures of suspected cases of enteric, and up to the end of August they recovered only *B. paratyphosus* B and *B. typhosus*. At this time they noticed a clinical modification of the (enterica) epidemic. Cases of gastric derangement with fever became less numerous and less severe, many were accompanied by "extension to the biliary passages," and numerous cases of jaundice occurred. Coincident with this modification in clinical type the nature of the microbes isolated by blood culture changed. The organisms they now recovered corresponded in general characters to paratyphoid bacilli, but would not agglutinate with either their paratyphoid A or paratyphoid B serums from the Pasteur Institute. They provisionally regarded it as a new type, *B. paratyphosus* "D" (= Dardanelles). They state that from nearly all their cases of jaundice, and from those of "gastric derangement with fever" during the jaundice epidemic they found no longer *B. typhosus* and *B. paratyphosus* B, but this atypical variety.

In a later paper (1916) the authors report the result of the examination of ninety-four of the aberrant strains at the Pasteur Institute. Eighty-four strains were declared to be *B. paratyphosus* A, six *B. paratyphosus* B, and four *B. typhosus*. However, MM. Sarraillhé and Clunet see no reason to modify their opinion that "camp jaundice is merely a hepatic manifestation of the paratyphoid epidemic."

The main epidemiological features described by MM. Sarraillhé and Clunet as occurring in the French lines were repeated at Lemnos, but the same close relationship between jaundice and paratyphoid A was not observed either clinically or in the bacteriological laboratories.

Captain Archibald and his colleagues (1916) at Mudros East found that in September and October the incidence of paratyphoid B steadily rose and was the prevailing type, but about the middle of November the number of cases of paratyphoid A shot up, while that of paratyphoid B rapidly fell, the typhoid cases, as before, remaining low. Our experience at No. 3 Australian General Hospital at Mudros West indicates a more gradual and later change of type. During October, November, and December the number of bacteriologically diagnosed cases of paratyphoid A was 83, 76, and 45; of paratyphoid B 34, 40, and 18 respectively, but after the third week in December only paratyphoid A was met with. Jaundice was first observed in September. In October the number of cases admitted rapidly increased. During the later weeks of October and early weeks of November it was extremely common amongst troops and hospital personnel stationed at Mudros, and numbers of patients contracted the disease in the hospital.

Judged by admissions to No. 3 Australian General Hospital, the epidemic of paratyphoid A was in full swing when jaundice appeared. Some patients suffering from the former undoubtedly had jaundice, but the same was the case with patients suffering from typhoid, paratyphoid B, and dysentery, and the impression made upon us was that we were in the presence of two widespread epidemics, one of which was occasionally superimposed upon the other. The same conclusion was arrived at by Lieutenant Campbell (1916) from his experience at Cape Helles.

The experience of MM. Sarrailhé and Clunet was evidently different from ours, and it is difficult to disprove their contention. I can only point out that the prevalent type of jaundice at No. 3 Australian General Hospital, which is described at the commencement of this paper, was not accompanied by a continuous fever, and that paratyphoid bacilli were not recovered from such cases, nor did their serums agglutinate *B. paratyphosus* A or B, although the strains of both of these organisms which we employed were readily clumped by the serums of patients suffering from these diseases. Instances such as they record did occur, but can, I submit, be more reasonably interpreted as double infections. Paratyphoid fever has been abundantly studied in different parts of the world since its first differentiation by Achard and Bensaude in 1896, but jaundice is surely a rare symptom, for I cannot discover a single recorded instance of its occurrence in the literature.

Another account of the disease given by Major Hurst (1916) seems to me to accept too readily one possible view of its pathology. According to this observer the jaundice is caused by an extension of a catarrhal process from the duodenum, and the enlargement of the liver " must be due to a general infection secondary to that of the alimentary canal." I am not clear exactly what Major Hurst had in mind in framing the first part of the sentence, but the latter portion is unmistakable. However, Major Hurst is evidently not entirely satisfied with this view, for on p. 106 he admits that it is uncertain whether the primary infection is duodenal or through the blood.

Willcox (1916) also inclines to the opinion that the epidemic jaundice of campaigns starts as a gastro-intestinal infection, and that cholangitis follows from spread from the duodenum This view leads both Willcox and Hurst to the conclusion that infection is conveyed by the alimentary tract.

It is with trepidation that I enter the arena against Lieutenant-Colonel Willcox and Major Hurst when the combat is over matters largely clinical, but it seems to me unfortunate that, in the absence of definite knowledge of the etiology of the disease, these gentlemen should thus light-heartedly throw the weight of their authority in favour of one possible interpretation of its pathology and method of spread. I submit that the picture presented by the infectious jaundice at Gallipoli, and the morbid anatomy, meagre as it is, are more consistent with the view that we are dealing with a hepatitis following a systemic infection than with that of a catarrhal jaundice from plugging of the bile ducts from extension of an inflammatory process from the duodenum.

The reasons which influence me in arriving at this conclusion are briefly:

1. The illness is ushered in by a febrile attack like influenza, but usually of shorter duration.
2. Jaundice does not occur for some days, and is preceded by swelling and tenderness of the liver.
3. Bile is rarely completely prevented from entering the intestine.
4. The spleen is often enlarged.
5. Albuminuria is not uncommon.
6. Notwithstanding the comparatively slight initial illness the existence of a serious toxaemia is indicated by lassitude, which endures for some weeks, and evidence of myocardial poisoning.
7. Histological evidence of necrosis of liver cells and inflammation around the portal areas.

Summary and Conclusions.

1. No parasites were discovered in blood films taken during the disease.
2. Blood cultures were sterile unless the jaundice supervened during an attack of typhoid or paratyphoid.
3. The observations made at No. 3 Australian General Hospital at Mudros do not support the conclusion of MM. Sarrailhé and Clunet at Cape Helles that the jaundice was merely a manifestation of paratyphoid fever.
4. Bacteriological analysis of duodenal contents removed from patients affords no justification for the view that the jaundice was due to a bacterial infection spreading up the bile ducts.
5. The livers of two patients who succumbed during the convalescent stage of jaundice showed microscopical evidence of hepatitis with necrosis of liver cells.
6. It is pointed out that the infectious jaundice of Gallipoli, although much milder, presents analogies to the severer form spirochaetosis icterohaemorrhagica, and it is contended that the symptomatology and morbid histology are consistent with the view that it is primarily a systemic infection.

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THE DISTRIBUTION OF TYPHOID AND PARATYPHOID INFECTION AMONGST ENTERIC FEVERS AT MUDROS, OCTOBER TO DECEMBER, 1915.

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(*Read before Anzac Medical Association, Cairo, February* 20, 1916.)

The early records from France of the incidence of enteric fever upon inoculated and uninoculated British troops showed no significant difference. This was in contrast with results previously obtained, and notably with those patiently collected over the years 1905 to 1908, by the War Office Committee appointed to examine into the virtues of anti-typhoid inoculation, according to whose report the incidence was about 1 to 9 respectively.[1]

This discrepancy might obviously be accounted for, if the prevailing type of enteric infection was not typhoid but paratyphoid, for Kabeshima[2] has shown that inoculation against typhoid affords no protection against paratyphoid A or B. Accordingly, endeavours were made towards the end of 1914 to differentiate these latter fevers from typhoid.

Early in 1915, Dreyer, Walker and Gibson[3] published the results of the examination of a limited series of cases at the base hospital at Oxford. They concluded that no case of typhoid fever had occurred in the inoculated individuals of their series, and that, as found by Kabeshima, paratyphoid fevers had occurred with equal frequency among anti-typhoid inoculated and non-inoculated individuals. Dreyer, Walker and Gibson (*loc. cit.*) were of opinion that the cases returned as typhoid fever amongst our troops were to a large extent paratyphoidal infections.

A large portion of our work at No. 3 Australian General Hospital at Lemnos consisted in helping our medical colleagues to arrive at an accurate diagnosis of the various intestinal diseases, which bulked so largely in the medical practice of the hospital. To this end, in the case of the enteric fevers, we chose the serological method as our routine procedure. It would not have been possible, with the material and assistance at our disposal, to have made more than one-fifth the number of observations by blood culture, and at least half of these would have miscarried, as so many of the patients were past the first week of the disease on admission.

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[1] Leishman, Colonel W. B. "Statistical Results of Anti-typhoid Inoculation," Journal of the Royal Army Medical Corps, xii, p. 163. 1909.

[2] Kabeshima T. "Ueber Typhus und Paratyphus Schutzimpfung, usw., in der kaiserlich. Japanischen Marine." *Centralblatt für Bakteriologie*, Originale Band 74, p. 294. 1914.

[3] Dreyer, G., Walker, E. W. A., and Gibson. A. G. "Typhoid and Paratyphoid Infection in relation to Anti-typhoid Inoculation." *Lancet*, February 13, 1915.

We were aware that the interpretation of agglutination results would, in the case of persons inoculated against typhoid, be a matter of some difficulty; but we had the advantage of working in close co-operation with our physicians, who kept us in touch with the subsequent history of the patients so that repeated observations could be made if desirable. We therefore hoped, that in the great majority of cases, any ambiguity might be removed.

There is no one perfect method for estimating the distribution of the various enteric infections. Conclusions drawn from blood cultures alone are not free from error, for the proportion of successful cultivation is higher in typhoid than in paratyphoid B fever.

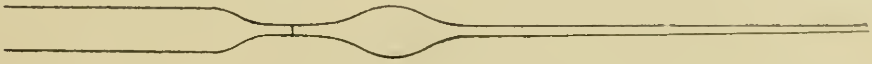
During the three months October to December, 1915, we gathered a considerable amount of data which we shall proceed to deal with, in view of its bearing upon the distribution of enteric fevers at Gallipoli during this part of the campaign.

In carrying out the agglutinations we had from time to time valuable help from Sister Williams and Captains Bahr, Brennan and Maclure. In scrutinizing the cases whose sera gave an agglutination to typhoid only, the importance of which scrutiny will be emphasized below, we had invaluable help from Lieutenant-Colonel Stawell, Lieutenant-Colonel de Crespigny and Captain Ercole. To one and all we express our deep sense of gratitude.

METHOD EMPLOYED IN DETAIL.

The emulsions were, in the first instance, made from strains of typhoid, paratyphoid A and paratyphoid B bacilli obtained from the Lister Institute. The first is a readily agglutinable strain which has been propagated in the laboratory for several years. The two last are descendants of Schottmüller's original strains. Subsequently we substituted a locally isolated strain of *B. paratyphosus* B, as the Schottmüller strain did not form good emulsions. The bacilli were grown for twelve hours on nutrient agar, emulsified in saline by gently agitating the bottles and diluted so as to show definite opalescence when placed in the tubes used for the agglutinations, which were about ¼ inch in diameter. Formalin was added to make a concentration of 0·1 per cent. Each brew of emulsion was tested against standard sera obtained from the Lister Institute, which agglutinated all three emulsions up to a dilution of 1 in 16,000. The agglutination was specific in a dilution of 1 in 50. Lower dilutions were not tried. As fresh emulsions were made from time to time, they were tested in the same way against the corresponding standard serum. No significant variation in agglutinability was observed during the course of the observations.

About a fifth of a cubic centimetre of blood was drawn from the patient into a sterile glass pipette and sealed. The blood serum was usually separated from the clot by centrifuging on the same day. For diluting the serum, pipettes drawn out in the flame and cut off at a point where their orifice had an external diameter of 1·12 millimetre were employed. Such a pipette drops 0·02 cubic centimetre of serum if held vertically. Two drops made up to one cubic centimetre affords a dilution of 1 in 25. For arranging the series of dilutions we used pipettes drawn so as to leave a small bulb intervening between the two capillary portions (fig. 1), which latter should have thick walls.



The contents of the pipette is arranged so as to equal about half the volume it is desired to work with in the agglutination tubes. The exact content is not material if the same pipette is used throughout the series of dilutions and subsequently for adding the emulsions. For convenience, however, we made two stock sizes of pipettes which contained 0·15 cubic centimetre and 0·25 cubic centimetre respectively. The pipettes in each series were then interchangeable.

The successive dilutions were made by the usual method, viz.: Two volumes of the first dilution of serum were placed in tube No. 1 of the series, and into tubes 2, 3, 4, etc., was placed one volume of water. Thereafter, one volume was removed from tube No. 1, emptied into tube No. 2 and mixed. A volume of this mixture was withdrawn and emptied into No. 3 and mixed. One volume withdrawn from No. 3 was emptied into No. 4, mixed, and so on until the last tube of the series was reached, when, after mixing, the one volume removed was discarded. In this way, each successive tube contained serum twice as dilute as the previous one. The procedure having been carried out in triplicate, to every tube in each series an equal volume of emulsion of typhoid, paratyphoid A, or paratyphoid B bacilli respectively, was added and the contents mixed. The tubes were placed in the incubator at 37° C. for two hours, allowed to remain on the bench for one hour and the results read.

The following conventional series of marks was awarded and will be employed in this communication :—

Complete agglutination	+++
Agglutination visible to the naked eye	++
Agglutination visible with lens magnifying four diameters ..	+
No agglutination visible with lens magnifying four diameters ..	−

The sera of 350 patients were tested against *B. typhosus*, *B. paratyphosus* A and *B. paratyphosus* B in dilutions of 1 in 100, 1 in 200, 1 in 400 and 1 in 800. In the remainder the 1 in 800 dilution was omitted to save labour as it was found that the results could, in most cases, be equally well read without it. After the first two months a lower dilution, 1 in 50, was employed for the paratyphoid A series, as we found that we were missing some infections due to this organism by beginning at 1 in 100. For reasons which will be entered into later these cases were generally recorded as typhoid.

TABLE I.

No agglutination (minimal dilution of sera 1 in 50 or 1 in 100) ..	163
Agglutination to *B. typhosus*	138
,, ,, *B. paratyphosus* A	66
,, ,, ,, and *B. typhosus*	136
,, ,, ,, *B. typhosus* and *B. paratyphosus* B (last slight or transient)	9
,, ,, ,, and *B. paratyphosus* B (latter slight or transient)	2
,, ,, *B. paratyphosus* B	19
,, ,, ,, ,, and *B. typhosus*	88
,, ,, ,, ,, *B. typhosus* and *B. paratyphosus* A (last slight or transient) ..	5
,, ,, ,, ,, and *B. paratyphosus* A (latter slight or transient)	1
	627

RESULTS.

Observations were made upon the sera of 627 patients and 151 normal persons. As we were functioning as the clinical laboratory of a large general hospital, the patients whose sera we examined were not all enterics. If the medical officer thought that a seralogical examination would help him towards a diagnosis, the blood was sent to the laboratory. The 627 patients consequently included a number who were suffering from other febrile diseases.

In Table I below are set forth the gross results.

The Interpretation of Agglutination Findings in Patients, previously Inoculated against Typhoid Fever.

(1) *Agglutination of B. typhosus.*—Dealing, as we were, with a population almost entirely inoculated against typhoid within fourteen months, it is not surprising that 376 out of the 627 should show an agglutination to *B. typhosus* in a dilution of 1 in 100 or upwards.

Before we are able to draw any conclusions from our results, it is first of all necessary to ascertain, following the technique used, the amount of agglutination which occurs from inoculation only. To determine this, the blood of members of the staff and hospital orderlies, who had not been sick, was examined under similar conditions to those employed in the case of patients.

Agglutination Titre for *B. typhosus* of the Sera of seventy-five healthy persons inoculated against Typhoid seven to fourteen months previously.

7 agglutinated	in a dilution of		1 in 800
12 ,,	,,	,,	1 in 400
22 ,,	,,	,,	1 in 200
16 ,,	,,	,,	1 in 100
18 did not agglutinate	,,	,,	1 in 100

That is, two-thirds showed an agglutination titre of 1 in 200 or upwards and in 10 per cent it was 1 in 800 or upwards.

In addition to the above experiment, the pooled sera of twenty-eight men inoculated on the same day fourteen months previously, was found to give a good agglutination at 1 in 200, a result in close agreement with the above.

It follows, therefore, that in an inoculated person an agglutination of *B. typhosus* by his serum up to the limits observed by us in patients, is not necessarily significant of typhoid infection, and that in order to arrive at a diagnosis of typhoid fever in our cases other means must be resorted to.

When we commenced our observations we were under the impression that the progressive development of typhoid agglutinins during the course of illness, reaching sometimes a titre of 1 in 1,000 or upwards, might, in the absence of any discoverable agglutinins to *B. paratyphosus* A or B, be taken as evidence of typhoid fever.

We were strengthened in this belief by the conclusions arrived at by Dreyer, Walker and Gibson (*loc. cit.*), who pointed out, quite rightly, that a differential diagnosis may be arrived at by measuring the agglutination titre to typhoid and paratyphoid bacilli respectively over a period of a few days. This method is, however, beset with pitfalls.

Early in our observations, as we shall proceed to show, it became apparent that many sera agglutinating one of the paratyphoid bacilli simultaneously agglutinated typhoid bacilli to an unexpected degree. Further, in a number of cases of paratyphoid fever in which repeated observations were carried out, a progressive development of typhoid agglutinins preceded by one to two weeks the development of agglutinins to the paratyphoid bacillus. We give below examples illustrating this phenomenon in which paratyphoid bacilli were isolated. Unless the observations had been persisted in, all of these would have been diagnosed as typhoid fever. We ought to have been alert to this possibility, for in 1911 Grattan and Harvey[1] described its occurrence in paratyphoid A fever in India. Attention was also drawn to it by Firth,[2] and later by Safford.[3]

			1 in 100	1 in 200	1 in 400	1 in 800
No. 3729	Before illness.	Ty	+ +	–	–	–
		A	–	–	–	–
		B	–	–	–	–
	After 1 week.	Ty	+ + +	+ + +	+ +	–
		A	–	–	–	–
		B	–	–	–	–
	After 2 weeks.	Agglutination the same. *B. paratyphosus* A was recovered from urine.				
	,, 3 ,,	Agglutination the same.				
	,, 4 ,,	,, ,,				
	,, 5 ,,	Ty	+ + +	+ + +	+	–
		A	+ + +	+ + +	+ + +	+ +
		B	–	–	–	–

B. paratyphosus A was isolated from the fæces in the fourth week.

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[1] Grattan, Major H. W., and Harvey, Major D. "An Inquiry into a small Epidemic of Paratyphoid Fever in a Camp in India," JOURNAL OF THE ROYAL ARMY MEDICAL CORPS, 1911, vol. xvi, p. 9.

[2] Firth, Colonel R. H. "Recent facts as to Enteric Inoculation and the Incidence of Enteric and Paratyphoid Fevers in India." JOURNAL OF THE ROYAL ARMY MEDICAL CORPS, 1912, xix, p. 157.

[3] Safford, Major A. H. "Paratyphoid Fever, an account of two epidemics, etc." JOURNAL OF THE ROYAL ARMY MEDICAL CORPS, 1913, xx, p. 567.

			1 in 100	1 in 200	1 in 400	1 in 800
No. 3681	1st week of illness.	Ty	–	–	–	–
		A	–	–	–	–
		B	–	–	–	–
	3rd week of illness.	Ty	+++	+++	++	+
		A	–	–	–	–
		B	–	–	–	–
	4th week of illness.	Ty	+++	+++	++	+
		A	++	+	–	–
		B	–	–	–	–

B. paratyphosus A was recovered from the sputum and pleural exudate during the third week of illness. The patient died in the fourth week with gangrene of the lung and localized pleurisy. *B. paratyphosus* A was recovered in pure culture from the spleen. The bile was sterile.

			1 in 100	1 in 200	1 in 400	1 in 800
No. 3778	1st week of illness.	Ty	–	–	–	–
		A	–	–	–	–
		B	–	–	–	–
	2nd week of illness.	Ty	++	+	–	–
		A	–	–	–	–
		B	–	–	–	–
	3rd week of illness.	Ty	+++	+++	++	+
		A	+++	+++	+++	+
		B	–	–	–	–

B. paratyphosus A was recovered from the fæces during the third week.

			1 in 100	1 in 200	1 in 400	1 in 800
No. 522	2nd week of illness.	Ty	+++	+	–	–
		A	–	–	–	–
		B	–	–	–	–
	3rd week of illness.	Ty	+++	+++	++	+
		A	–	–	–	–
		B	–	–	–	–
	5th week of illness.	Ty	+++	+++	++	+
		A	–	–	–	–
		B	+++	+++	+++	+++

The patient died in the sixth week of illness. A pure culture of *B. paratyphosus* B was isolated from the spleen and bile. The heart blood taken post mortem agglutinated *B. paratyphosus* B in a dilution of 1 in 10,000.

In the instances detailed above, infection with one of the paratyphoid organisms stimulated the production of typhoid agglutinins early in the disease, and it was not until as late as the third to fifth week that the titre for the infecting organism rose to 1 in 100. This occurred in about two per cent of our cases, all, with one exception, paratyphoid A fever.

As soon as we became aware of these facts we regarded with suspicion the development of typhoid agglutinins in a patient previously inoculated against typhoid fever, and he was only diagnosed as typhoid when *B. typhosus* was isolated, or, being clinically a case of one of the enteric group of fevers, when no evidence of paratyphoid infection could be obtained. Even so, we have reason to believe that some cases of infection with *B. paratyphosus* A were returned as typhoid, for, occasionally, the development of agglutinins to this organism is slight and transient.

In order to throw some further light upon the interpretation of the rise in typhoid agglutinins in paratyphoid infections, we inoculated healthy persons, some of whom had previously received anti-typhoid vaccine, with paratyphoid A and B bacilli. In about half of those who had been previously inoculated this was followed by a considerable development of typhoid agglutinins. The instances given below were amongst the most striking:—

			1 in 100	1 in 200	1 in 400	1 in 800
No. 1001	Inoculated against typhoid six months previously	Ty	–	–	–	–
		A	–	–	–	–
		B	–	–	–	–
	Eleven days after injection of 200 millions *B. paratyphosus* A and 200 millions *B paratyphosus* B	Ty	+++	+++	+++	++
		A	+++	+++	+++	+++
		B	+++	+++	+++	++
No. 1002	Inoculated against typhoid five months previously	Ty	–	–	–	–
		A	–	–	–	–
		B	–	–	–	–
	Ten days after 200 millions each of *B. paratyphosus* A and B	Ty	+++	+++	++	–
		A	+++	+++	+++	+++
		B	++	–	–	–
	Fifteen days after above	Ty	+++	+	–	–
		A	+++	+++	+++	+++
		B	++	–	–	–
No. 1008	Inoculuted against typhoid twelve months previously	Ty	–	–	–	–
		A	–	–	–	–
		B	–	–	–	–
	Seven days after inoculation with 50 millions of each *B. paratyphosus* A and B	Ty	+++	+++	+++	+++
		A	+++	+++	+++	++
		B	+++	++	+	–

No. 1010	Inoculated against typhoid six months previously	Ty	++	–	–	–
		A	–	–	–	–
		B	–	–	–	–
	Eleven days after second inoculation with 500 millions of each *B. paratyphosus* A and B	Ty	+++	+++	+++	+++
		A	+++	+++	+++	+++
		B	+++	+++	++	+
No. 1012	Inoculated against typhoid seven months previously	Ty	++	–	–	–
		A	–	–	–	–
		B	–	–	–	–
	Fifteen days after inoculation with 50 millions *B. paratyphosus* A	Ty	+++	+++	+++	++
		A	+++	+++	+++	+++
		B	–	–	–	–
No. 1013	Non-inoculated.	No agglutination to Ty. A or B in 1 in 100				
	Ten days after inoculation with 200 millions each of *B. paratyphosus* A and B	Ty	–	–	–	–
		A	+++	+++	+++	++
		B	+++	+++	++	–
No. 1014	Non-inoculated.					
	Nine days after 200 millions each of *B. paratyphosus* A and B	Ty	–	–	–	–
		A	+++	+++	++	+
		B	+++	++	–	–
No. 1015	Non-inoculated.					
	Ten days after 250 millions each of *B. paratyphosus* A and B	Ty	–	–	–	–
		A	+++	+++	+++	+++
		B	++	+	–	–

Harvey and Wood[1] found in the cases of paratyphoid A fever which they studied, that the agglutinins for both *B. typhosus* and *B. paratyphosus* A were absorbed by an emulsion of the latter, whereas an emulsion of typhoid bacilli only absorbed the homologous agglutinins. According to this observation, we are merely dealing with an example of group agglutination. Co-agglutinins were certainly produced in some of our patients, but we doubt whether this is the whole explanation, as it did not occur when individuals not inoculated against typhoid were injected with a vaccine containing only *B. paratyphosus* A and *B. paratyphosus* B. We also tried to absorb the agglutinins for typhoid bacilli in two instances, where they were well developed, by shaking out with a strong mixed emulsion of paratyphoid A and B bacilli, but only succeeded in reducing them. The development of well-marked agglutination to typhoid bacilli weeks before the specific agglutinins arrived, suggest another interpretation, namely, that in an inoculated person, the mechanism for the manufacture of agglutinins being already laid down, the introduction of paratyphoid organisms stimulates this mechanism to further activity.

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[1] Harvey and Wood. Quoted by Firth, JOURNAL OF THE ROYAL ARMY MEDICAL CORPS, xix, p. 163.

In the same report from which the above excerpt is taken it is also stated that "in the case of a man who has been inoculated against enteric and whose serum gave a positive reaction to the *B. typhosus*, absorption with *B. paratyphosus* A will only reduce the agglutinin titre as it cannot remove the agglutinins due to inoculation which are specific for the *B. typhosus*." See Firth, JOURNAL OF THE ROYAL ARMY MEDICAL CORPS, 1911, vol. xvii, p. 136.

(2) *Agglutination of B. paratyphosus A or B. paratyphosus B or both of these Organisms.*—The serum of 326 cases agglutinated *B. paratyphosus* A or B in dilutions of 1 in 100 and upwards. In 238 of these, more or less agglutination of *B. typhosus* was exhibited also. Nevertheless, for reasons entered into above, in individuals inoculated against typhoid, these were returned as paratyphoid A or B fevers respectively and, whenever opportunity offered of confirming this by isolation of the infecting organism, the conclusion was found to be justified. With the dilutions we employed, in ninety-five per cent of the cases the results were unequivocal, but in 17 out of a total of 326 the serum agglutinated both *B. paratyphosus* A and *B. paratyphosus* B. The agglutination generally occurred in considerably higher dilution with one than with the other, so as to leave little doubt as to the diagnosis. In a few cases this was not so, and it was impossible to say from one examination which was the infecting organism, and later observations at intervals of about a week were made. At the same time efforts were made to recover the organism responsible for infection.

The following are examples illustrating how the diagnosis was cleared up by a second examination of the blood:—

			1 in 100	1 in 200	1 in 400	1 in 800
No. 538	6.11.15	Ty	+++	+++	+++	+++
		A	+++	++	–	–
		B	+++	+++	+++	+++
	18.11.15	Ty	+++	++	–	–
		A	–	–	–	–
		B	+++	+++	+++	+++

B. paratyphosus B was recovered from the fæces.

			1 in 100	1 in 200	1 in 400	1 in 800
No. 449	23.10.15	Ty	+	−	−	−
		A	+++	+++	+++	+++
		B	++	++	+	−
	1.11.15	Ty	+	−	−	−
		A	+++	+++	+++	+++
		B	−	−	−	−

B. paratyphosus A was recovered post mortem.

			1 in 100	1 in 200	1 in 400	1 in 800
No. 548	7.11.15	Ty	++	+	−	−
		A	+++	+++	+++	+++
		B	+++	+++	++	+
	16.11.15	Ty	+	−	−	−
		A	+++	+++	+++	+++
		B	+	+	−	−
No. 379	16.10.15	Ty	+	−	−	−
		A	++	+	+	+
		B	+	+	+	+
	15.11.15	Ty	−	−	−	−
		A	+++	+++	+++	+++
		B	++	+	−	−

In making a diagnosis we have regarded the organism for which agglutinins persisted, as responsible for the infection. In those cases in which we succeeded in isolating the causal organism this view was justified.

The amount of co-agglutinins present at an early stage of the illness in five per cent of patients with paratyphoid fevers is noteworthy. Compared with our standard sera, made by injecting rabbits with a laboratory strain of paratyphoid A or B bacilli, it is considerable. It must be remembered, however, that the standard sera were made from strains selected on account of their specificity, whereas the " wild " strains occurring in nature are not always so specific. Further, it is probable that man is not as good an animal to use for the manufacture of highly specific sera for this group of organisms, resembling the horse in this respect rather than the rabbit.

ANALYSIS OF OBSERVATIONS.

We are now in a position to proceed to the analysis of our observations and see what indications they afford as to the distribution of different enteric fevers at Gallipoli. The 163 instances in which the serum gave no agglutination in the minimum dilution used, 1 in 100, may be summarily dismissed as they furnish no information. They comprise cases in which the serum was sent for examination too early in the disease and others in which the subsequent clinical history indicated that the patient was suffering from some disease other than one of the enteric fevers. The total number of cases in which agglutination with one or more of the bacilli of the enteric group was observed was 464. Of these, 213 sera agglutinated *B. paratyphosus* A and are, for reasons given above, regarded as significant of paratyphoid A infection. For parallel reasons, we regard 113 as infections by paratyphoid B.

The determination of the number of typhoid infections is not so simple.

The 138 cases in whose sera only typhoid agglutinins were discovered were not necessarily typhoid fevers, for all but five of the patients had been inoculated. These five exceptions we place to the credit of typhoid infections on the agglutination findings. In seven other cases we succeeded in isolating *B. typhosus* from the blood or excreta either during life or after death, leaving 126 cases in which serological observations might be interpreted either by previous inoculation or by recent infection.

Medical officers were in the habit of sending blood to the laboratory for serological examination whenever they suspected that they might be dealing with a case of one of the enteric fevers and sometimes irrespective of the time the patient had been ill. Consequently, there are in this group a number of cases which subsequently proved to be dysentery, pneumonia, malaria, influenza, or relapsing fever. It also includes thirty-five cases of epidemic jaundice in which serological observations were deliberately made, as there was a widespread impression at first that the disease was associated with infection by a paratyphoid organism. We may eliminate these thirty-five cases of jaundice from further consideration, as, whatever the infective agent may have been, there is no evidence that it was due to a paratyphoid bacillus.

The remaining ninety-one cases were either investigated at the time, or their records carefully scrutinized for us by Lieutenant-Colonel Stawell, Consulting Physician to our Unit, and we take this opportunity of expressing to him our exceeding gratitude. Without his assistance in this matter it would not have been possible to draw what is, perhaps, the most important conclusion from our observations, namely, the relative prevalence of true typhoid fever at Gallipoli. After submission to his scrutiny, these ninety-one cases which might possibly have been typhoid infections were reduced to thirteen. This number includes all those which, on clinical grounds, might have been cases of one or other of the enteric fevers. It is certainly a maximal estimate for, as mentioned above, we have reason to believe that some cases of paratyphoid A fever, which the method we employed failed to discover, are included in it.

The results of the analysis of our observations, after this eliminative procedure has been applied to the cases whose sera agglutinated only *B. typhosus*, are as follows:—

Cases of typhoid fever	.. ..	25	..	7 per cent
,, ,, paratyphoid A fever	..	213	..	61 ,,
,, ,, paratyphoid B fever	..	112	..	32 ,,
Total of enteric fevers	..	350		

The patients at No. 3 General Hospital, A.I.F., were derived from Gallipoli and the lines of communication. Imperial troops and Australians were indiscriminately admitted. As the sample covered by our observations was of fair size, we shall not commit a serious error if we apply our conclusions to the distribution of enteric fevers at the Dardanelles.

From information kindly furnished us by Lieut.-Colonel Aldridge, R.A.M.C., there were 5,700 cases returned as enteric fevers from this area from the commencement of the campaign until the middle of December, 1915. According to a statement made by the Under Secretary for War in the House of Commons on December 23, the total medical casualties during the same period were 96,683.

If we further assume that the distribution we have arrived at is applicable to the whole of this period, the invaliding rate from typhoid fever represents less than half per cent of the total sickness during the period.

This highly satisfactory result must, presumably, be attributed to anti-typhoid inoculation, for the conditions were not unfavourable to the spread of enteric disease.

As soon as we became aware from our observations that the great proportion of the cases of enteric fevers amongst Australian troops was due to paratyphoid infections, we represented the facts to the Director of Medical Services of the Commonwealth Forces and advised that Australian troops should be at once inoculated with a vaccine containing paratyphoid and typhoid bacilli. This recommendation was approved by the Minister for Defence and early this year all Australian Units were so inoculated.

Henceforth, the interpretation of observations upon the agglutination of enteric organisms will, in the case of patients belonging to Australian units, be too difficult to possess any practical value and the isolation of the infecting organism must be resorted to for diagnosis.

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No 14

NOTES ON THE VACCINATION OF GUINEA-PIGS WITH B. PERFRINGENS.

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THE following paper is a brief account of certain vaccination experiments carried out with Bacillus perfringens (B. aerogenes capsulatus). The animals used were guinea-pigs. Before considering the experiments with vaccinated animals it will be useful to give an account of the pathogenicity of B. perfringens for guinea-pigs.

Different strains of B. perfringens vary very markedly in virulence and the same strain may vary according to cultural conditions. It was found that a 24 to 48-hour broth culture gave a fairly uniform result in regard to infectivity, though fluctuations do occur. The infectivity of the organism was observed to be relatively very slight when inoculated in the form of a saline emulsion from a nutrient glucose agar slope. Moreover, saline emulsions of sporing cultures grown on inspissated serum failed to infect upon the two occasions on which this method was used, although the dose injected was in one case equal to three slopes emulsified and in the other equal to two slopes.

In determining the lethal dose and in all the infecting inoculations broth cultures of 24 to 48 hours were used. The animals were inoculated into the muscular tissue of the thigh, care being taken to cause no further injury than that occasioned by the insertion of the needle.

In vaccination experiments care must be taken to fix the lethal dose within fairly narrow limits. The lethal dose for a guinea-pig of 250 to 300 grammes was about 60 to 100 million organisms from a 24 to 48-hour broth culture of the more virulent strains. Four strains were used, of which S.R. 12, S.R. 8, and S.R. 9 were of very similar virulence; the fourth strain K. 13 was rather less virulent. Five million organisms of the more virulent strains failed to kill; one guinea-pig was killed with 30 millions, though its companion survived. Even at the 60 to 100 million dose a certain number survive; thus out of 52 guinea-pigs which received 60 to 100 millions of a living broth culture, 5 recovered, which gives a recovery rate of 9·6 per cent.

It should be noted that sometimes owing to some condition of growth which is not evident a strain may be less virulent for a time; thus on one occasion a group of guinea-pigs received 60 millions of a strain which usually kills at this dose, all the animals reacted but none died. This group is not included among the 52 animals cited above. It is, of course, obvious that the recovery rate is greater when the dose used is on the border-line of being lethal.

For the purposes of these experiments therefore it was concluded that 60 to 100 million organisms from a 24 to 48-hour broth culture of a virulent B. perfringens might be taken as the lethal dose for a guinea-pig of 250 to 300 grammes, but that in view of alterations in the virulence it is absolutely essential that every series of experiments be separately controlled. These doses refer to the particular strains used and to their condition during the period in question, and are probably not more than merely a general indication of the amount necessary to kill when pathogenic strains are used.

The following experiments were carried out to see if vaccination with B. perfringens afforded any protection against subsequent inoculation of a lethal dose.

Experiment A, of August 5th. 8 guinea-pigs (Nos. 94, 95, 96, 97, 100, 101, 102, and 103) vaccinated by injection of 3 doses four days apart of heated saline emulsion on July 21st, 24th, and 27th; doses 20 millions, 100 millions, and 1000 millions respectively of B. perfringens (S.R. 9 xxi.); vaccination complete on July 27th. August 5th: Infecting dose of 60 millions (S.R. 9 xxi.) living from broth culture injected. 3 guinea-pigs died within 18 hours, 3 died within 40 hours, and 1 died within 64 hours. 1 survived after severe reaction.

5 control guinea-pigs had same dose (60 millions living S.R. 9 xxi. from broth culture). 1 died within 18 hours, 2 died within 40 hours, and 1 died within 64 hours; 1 survived.

Experiment B, of August 12th. 5 guinea-pigs vaccinated. Saline emulsion heated S.R. 8 xviii: 27/7/15, 50 millions; 30/7/15, 150 millions; 2/8/15, 1000 millions. 12/8/15: Inoculated, infecting dose 60 millions of living bacilli from broth culture of S.R. 9 xxi. All the 5 guinea-pigs reacted and 3 had gangrene, but none of them died.

3 control guinea-pigs had 12/8/15 same dose, 60 millions, of S.R. 9 xxi. from broth culture. All the 3 control guinea-pigs survived; one had gangrene, one had œdematous swelling, one did not react.

In this case the infecting dose seems to have been sublethal.

Experiment C, of Oct. 6th. 25 guinea-pigs vaccinated with 1 dose of heated but not completely killed broth culture, 750 millions of S.R. 9 xxi. on August 7th. On Oct. 6th they were injected with 100 millions of S.R. 9 xxi. from a living broth culture. 20 guinea-pigs died within 21 hours, 2 died within 45 hours, 1 died within 69 hours, and 1 died within 90 hours. 1 guinea-pig survived.

Experiment D, of Oct. 6th. 5 guinea-pigs (Nos. 114 to 118) vaccinated with 3 doses at intervals of four days with saline emulsion from glucose-agar slopes of S.R. 12 xxvi.; emulsion heated but not completely killed; doses 50 millions, 150 millions, and 1000 millions respectively. Vaccination complete on August 2nd. On Oct. 6th 100 millions from living broth culture of S.R. 9. xxi. injected. 2 guinea-pigs died within 20 hours, 2 died within 28 hours, and 1 died within 69 hours.

Experiment E, of Oct. 6th. 3 guinea-pigs (Nos. 109, 111, and 113) vaccinated with 3 doses S.R. 8 xviii., heated but not entirely killed, saline emulsion four days apart; doses 50 millions, 150 millions, and 1000 millions respectively. Vaccination complete August 2nd. On Oct. 6th 100 millions from living broth culture of S.R. 9 xxi. injected. 2 guinea-pigs died within 20 hours and 1 died within 45 hours.

Experiment F, of Oct. 6th. 5 guinea-pigs (Nos. 104 to 108) vaccinated with 3 doses four days apart; doses 50 millions, 150 millions, and 1000 millions saline emulsion of strain S.R. 8 xviii. from glucose-agar slope heated but not completely killed. Vaccination complete August 2nd. On August 12th injection of living broth culture 60 millions of strain S.R. 9 xxi. They all showed swelling of leg and abdomen and 3 of them had gangrene, but all recovered. On Oct. 6th 100 millions from living broth culture of S.R. 9 xxi. injected. 1 guinea-pig died in 20 hours, 2 died in 24 hours, and 1 died in 45 hours. 1 guinea-pig survived.

Experiment G, of Oct. 6th. 3 guinea-pigs (Nos. 154, 155, and 156) inoculated 12/8/15 with 60 millions from a living broth culture of S.R. 9 xxi.; all recovered. On Oct. 6th 100 millions from a living broth culture of S.R. 9 xxi. injected. 1 guinea-pig died in 21 hours and 1 died in 45 hours. 1 guinea-pig survived.

Experiment H, of Oct. 6th. Guinea-pig 90 inoculated 17/7/15 with 60 millions living S.R. 9 xxi.; had bad gangrene fourth day; recovered. Reinoculated 6/10/15 with 100 millions living S.R. 9 xxi. from broth culture. Died in 48 hours.

Guinea-pig 99 vaccinated 21/7/15 with 20 millions heated S.R. 9 xxi.; no reaction. Vaccinated 24/7/15 with 100 millions heated S.R. 9 xxi.; no reaction. Vaccinated 27/7/15 with 100 millions heated but not completely killed of S.R. 9 xxi.; had œdema and gangrene but recovered. Inoculated 6/10/15 with 100 millions from a living broth culture of S.R. 9 xxi.; died in 28 hours.

Guinea-pig 102 vaccinated as for No. 99; had œdematous swelling but no gangrene. Inoculated 5/8/15 with 60 millions from living broth culture of S.R. 9 xxi.; had bad perfringens œdema, but recovered without gangrene. Inoculated 6/10/15 with 100 millions living broth culture of S.R. 9 xxi.; died in 24 hours.

Control to Experiments C, D, E, F, G, and H, of Oct. 6th, 1915. 10 normal guinea-pigs inoculated with living bacilli from broth culture of S.R. 9 xxi. on Oct. 6th. 7 guinea-pigs died within 21 hours and 3 died within 45 hours.

Experiment I, of 17/7/15. Guinea-pig 21 inoculated 2/6/15 with ½ c.c. of 48-hour living broth culture of K. 13 B—about 100 to 150 million organisms; œdema, gangrene by ninth day, recovered. Inoculated 17/7/15, with 60 millions of S.R. 9 xxi. from living broth culture. Guinea-pig very ill, œdema, gangrene on fourth day, animal recovered.

Guinea-pig 33 inoculated 16/6/15 with 110 millions from living broth culture K. 13 B; severe œdema, gangrene on eighth day, animal recovered. Inoculated 17/7/15 with 60 millions from living broth culture of S.R. 9 xxi.; guinea-pig died with bad œdema in 90 hours.

Experiment J, of 17/7/15. Guinea-pig 34, inoculated 16/6/15 with 340 million organisms K. 13 B in saline emulsion from 48-hour glucose-agar slope culture; no perceptible reaction whatsoever. Inoculated 17/7/15 with 60 millions organisms from 48-hour living broth culture of S.R. 9 xxi.; died in 18 hours.

Guinea-pig 41, inoculated 25/6/15 with saline emulsion from three slopes of sporing inspissated serum culture of K. 13 B; very slight swelling of limb. Inoculated 17/7/15 with 60 millions organisms from 48-hour living broth culture of S.R. 9 xxi.; died within 90 hours.

Guinea-pig 44, inoculated 25/6/15 with saline emulsion from two slopes of sporing culture inspissated serum of K. 13 B; very slight reaction. Inoculated 17/7/15 with 60 millions from living 48-hour broth culture of S.R. 9 xxi.; had bad œdematous swelling, but recovered.

Experiment K, of 17/7/15. Guinea-pig 55, inoculated 10/7/15 with 5 millions organisms from 48-hour living broth culture of S.R. 9 xxi.; very slight reaction. Inoculated 17/7/15 with 60 millions organisms from living broth culture of S.R. 9 xxi.; died in 90 hours.

Guinea-pig 56, inoculated 10/7/15 with 5 millions organisms from living broth culture of S.R. xxi.; very slight reaction. Inoculated 17/7/15 with 60 millions organisms from living broth culture 48-hours growth of S.R. 9 xxi.; died in 66 hours.

Experiment L, of 17/7/15. Three guinea-pigs (Nos. 51, 52, and 53) inoculated, 2/7/15 with 5 c.c. filtered broth culture of ten days' growth of K. 13 B; swelling at site of injection lasting two days. Inoculated 17/7/15 with 60 millions organisms from living broth culture of S.R. 9 xxi., 48 hours' growth. One guinea-pig died in 62 hours, 1 died in 66 hours, and 1 survived.

Controls to experiments of 17/7/15. 15 guinea-pigs (Nos. 76 to 90). Inoculated with 60 millions organisms from living broth culture of 48 hours' growth of S.R. 9 xxi. Five guinea-pigs died within 36 hours, 5 died within 60 hours, and 2 died within 84 hours; 3 recovered.

Experiments F, G, and H are of particular interest. In Experiment F the animals (which are the vaccinated animals of Experiment B) were first vaccinated with three doses of B. perfringens vaccine and then received a slightly sub-lethal dose of a living broth culture, which produced a quite typical B. perfringens illness, from which they recovered. Upon subsequent inoculation with a lethal dose of living broth culture 4 guinea-pigs died and 1 survived. In Experiment G the 3 guinea-pigs are the control animals from Experiment B. They had survived a slightly sub-lethal dose of living organisms, but upon being re-inoculated with a lethal dose of living B. perfringens 2 guinea-pigs died and 1 survived. It should be noted that the surviving animals in all these experiments showed a severe œdema which in certain cases culminated in gangrene.

Guinea-pigs 90 and 102 from Experiment H are interesting in that they show that the recovery from a typical severe B. perfringens illness does not protect against a subsequent infection. Experiment I may also be mentioned in this connexion. Thus, guinea-pig 21 recovered from a bad B. perfringens illness, and 6 weeks later, upon re-inoculation with the living bacillus, repeated the illness in all its symptoms and again recovered; the nature of the second attack was in no way different from the first. Guinea-pig 33 had a bad B. perfringens illness from which it recovered, and one month later was again inoculated with the living bacillus, whereupon the illness was repeated and the animal died.

In the Experiments I, J, K, and L of 17/7/15 there were 10 guinea-pigs which, having had previous inoculation with B. perfringens in some form or its growth products, were then subjected to a lethal dose of the living bacillus, and out of these 10 there were 3 which survived, which is equal to 30 per cent. Of the controls to this series, 3 out of 15 survived, being equal to 20 per cent. Upon such small numbers this difference does not seem to be very significant.

Considering the results of Experiments A, C, D, and E, which are, strictly speaking, the vaccinated animals, out of 41 guinea-pigs vaccinated as detailed above, 2 survived the inoculation of a lethal dose of living bacilli, which is equal to 4·8 per cent. If the figures of all the guinea-pigs are considered which received previous treatment of any kind with B. perfringens or its products (Experiment B is omitted), out of 62 guinea-pigs so treated 7, or 11·2 per cent., survived the inoculation of a lethal dose of living bacilli. While out of the 30 control guinea-pigs 4 survived the lethal dose, that is, 13·3 per cent. It must be observed that, taking the figures of the previously treated animals in that way, there are included a few cases in which the treatment amounted to a selection of a naturally more resistant individual (viz., Nos. 90, 21, and 33). It may further be recalled that in determining the lethal dose of living B. perfringens it was found that 9·6 per cent. of unprotected guinea-pigs survived the dose.

The general conclusion from the experiments detailed above is that previous vaccination with killed or attenuated cultures of B. perfringens does not cause any appreciable raising of the resistance of guinea-pigs against a subsequent lethal dose of living bacilli. Recovery from a previous infection with the organism does not prevent a repetition of the illness upon re-inoculation with living bacilli, nor does it apparently in any way alter the symptoms or influence the course of the disease.

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No 15

TYPHUS FEVER: AN EXPERIMENT ON VACCINATION WITH A COCCUS DERIVED FROM HUMAN CASES.[1]

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In July 1914 there occurred a small outbreak of typhus fever in Belfast. Dr. Penfold isolated a coccus from the blood and from the urine of two patients during the acute stage of the disease, and the same coccus from the urine of a convalescent (1916 [2]). It was impossible to make any deductions as to the causal connection between the coccus and the disease, from the data available in this work. The slight fevers produced by Dr. Penfold in two monkeys by the injection of freshly isolated strains of the coccus were not sufficiently characteristic to be convincing, and this reaction could not be repeated with later subcultures although the organism was grown continuously on human blood agar.

The work described in the following paper was therefore undertaken to test the connection of the organism with the disease, and also with the view of investigating the antigenic properties of Dr. Penfold's coccus as an immunizing agent against typhus. This investigation was made at Dr. Penfold's suggestion; he furnished the strains with which the immunization was carried out, and I am indebted to him not only for the general scheme of the work but also for much kind advice during its progress.

The premises of the experiment were as follows. From the work of Anderson and Goldberger, Nicolle, and others it was clear that the intraperitoneal injection of blood from a typhus patient into a healthy monkey gave a characteristic reaction. It had also been established that a substantial immunity was conferred upon monkeys by one injection of active blood; in addition, the bites of infected lice were shown to have a similar power of conferring immunity against subsequent typhus injection, although the monkeys so bitten did not show any febrile reaction. From these results it could be concluded that the subcutaneous injection of the virus of typhus was capable of producing an immunity in monkeys against the subsequent intraperitoneal injection of virulent typhus blood from a typhus patient.

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[1] Received December 6, 1916.

It was therefore suggested that if typhus were due to the coccus discovered by Dr. Penfold in the patient's blood, a vaccine made from this organism would immunize monkeys against a subsequent injection of virulent typhus blood.

The method of studying the typhus reaction in the monkeys was the usual one, namely, by taking the temperature of the animals twice daily and noting the occurrence and duration of the febrile period. This method is fairly sound, provided it is handled with sufficient criticism and care. The species of monkey must be taken into account and the nature of the temperature chart of the individual animal is of the utmost importance. It is quite necessary to have accurate knowledge concerning the temperature readings of the particular creature for some time previous to the inoculation of the test material. A considerable proportion of the monkeys sold in England are not in a suitable state for accurate experimental work of this kind, as they are running febrile charts due to causes entirely outside the experiment. In addition, care must be taken to keep the room temperature suitably warm and constant. And, finally, the actual handling of the monkey requires discretion, in order that the animal should not be unduly excited.

To illustrate the casualties that have to be contended with it may be mentioned that out of a total of twenty-two monkeys, six died from causes in no way connected with the experiment. Two other monkeys were unsuitable for use, owing to the fact that they were showing febrile charts on their own account.

The charts of the monkeys used for Experiment A are given over the whole period and are considered on their merits in the description of the experiments. As regards regularity of temperature it would appear that *Macacus rhesus* (when healthy) is one of the best species of monkey for experimental purposes.

EXPERIMENT A.

Six monkeys (Nos. 1, 8, 9, 4, 7 and 21) were selected; of these—1 (*Cercopithecus patas*), 8 (*Macacus cynomolgus*) and 9 (*Macacus rhesus*) were vaccinated with Dr. Penfold's coccus in the following manner:—

February 7, 1916.—Subcutaneous injection of a saline emulsion of about 150 million cocci, killed by adding 0·5 per cent. phenol (freshly prepared vaccine).

February 11, 1916.—Subcutaneous injection of a saline emulsion of about 1000 million cocci, killed by adding 0·5 per cent. phenol (vaccine, 5 days old).

February 2, 1916.—Subcutaneous injection of a saline emulsion of about 10,000 million cocci, killed by adding 0·5 per cent. phenol (freshly prepared vaccine).

February 21, 1916.—Subcutaneous injection of a fresh living emulsion containing 2000 million cocci.

The numbers of the organisms were counted under the microscope with dark ground illumination in a Thoma-Zeiss counting slide. The monkeys showed no local reaction and no appreciable general reaction. On 5th April, that is forty-four days after the last vaccinating injection, all the monkeys were tested by the inoculation of blood from a patient suffering from typhus fever (Case 1).

The patient, æt. 75, a man, took ill on Tuesday, 30th March 1916. Seen by doctor, Sunday, 2nd April, temperature 103° F. Brought into hospital, Tuesday, 4th April, temperature 100° F. Case visited Wednesday, 5th April, at 1.30 p.m., when a sample of the patient's blood was taken; the patient was very ill, his temperature 98° F. The rash appeared on Sunday, 2nd April, and was still very marked on the 5th. The case ended fatally. The family of the patient had yielded three previous cases of typhus during this outbreak.

Blood was drawn into an equal volume of sodium citrate solution at 1.30 p.m. on 5th April, and brought from the hospital in Kildysart, Co. Clare, to Dublin, and the monkeys were inoculated into the peritoneum at 8 p.m., that is, six and a half hours after the taking of the sample.

Monkeys 1 and 4 each received, intraperitoneally, 3 c.c. of citrate and blood mixture.

Monkeys 8 and 7 each received, intraperitoneally, 2½ c.c. of the blood citrate mixture.

Monkey 9 received, intraperitoneally, 3 c.c. of the blood citrate mixture.

Monkey 21 received, intraperitoneally, 4 c.c. of the blood citrate mixture.

The reaction of the monkeys was as follows, as shown by the temperature charts. The charts of all the monkeys were kept from 5th February (two months previous to the inoculation of the typhus blood) until 2nd May (that is, until the twenty-seventh day after the inoculation of the typhus blood).

Monkey 1 (*Cercopithecus patas*): vaccinated.—Rather an irregular chart (Fig. 1), with a tendency to a low morning temperature. From the 27th of March to 2nd April the temperature was markedly subnormal, the normal range had been regained at the time of inoculation of the typhus blood. Incubation period of five days during which the morning dip in the temperature is eliminated. On the sixth, seventh, and eighth days there is a slight rise in temperature. On the ninth day there is a sharp rise, fever is maintained till the fourteenth day. The temperature drops suddenly on the fifteenth and sixteenth days, thereafter the monkeys show so low a temperature that the clinical thermometer failed to give any reading. Finally, the animal died on 8th May.

Monkey 8 (*Macacus cynomolgus*): vaccinated.—This monkey shows a pretty level chart (Fig. 2); the daily variation is, however, as a rule, rather marked, being not infrequently as much as 2°. Incubation period of five days, slight rise on the sixth day, definite febrile reaction on the seventh day, rising sharply to 105°·8 F. on the eighth day; the fever is maintained through the thirteenth day. The temperature begins to come down on the fifteenth day, and declines gradually until, from the nineteenth day on, the chart has regained its original limits, the evening temperature running at about 101° F. The monkey was never really very ill, it kept fairly lively but took less food.

Monkey 9 (*Macacus rhesus*): vaccinated.—This monkey runs a very steady chart (Fig. 3) showing only slight variations. The incubation period is five days, there is a very slight rise on the sixth day, a sharp rise on the seventh day, and on the tenth day the fever is very marked, the temperature being 106° F. The febrile condition is maintained through the thirteenth day. The morning temperature on the fourteenth day is approximately normal, and after a slightly subnormal reading on the fifteenth day the chart regains very nearly its usual character by the sixteenth day. The monkey gave signs of malaise, but was not very ill in spite of the high temperature shown.

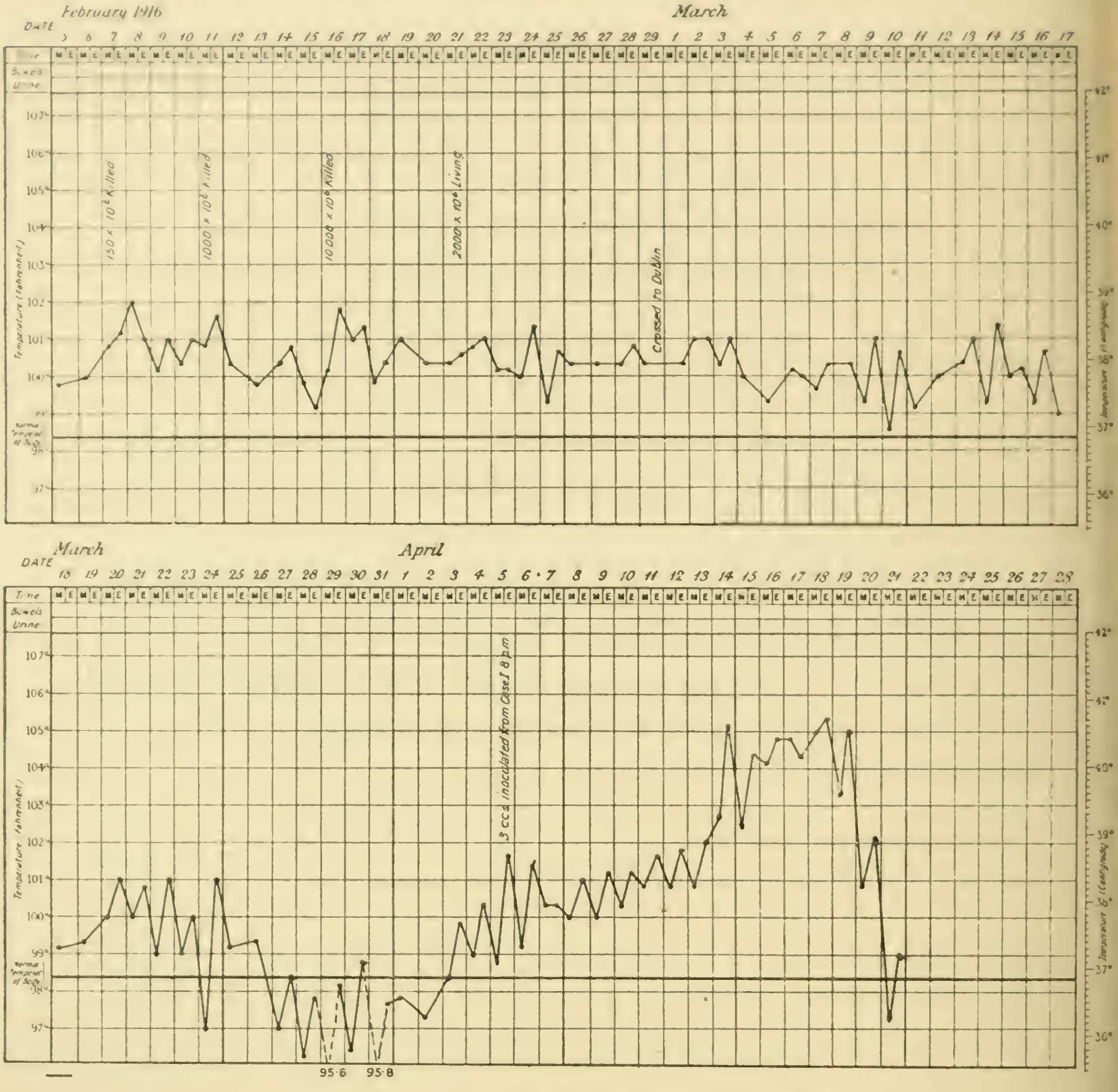

FIG. 1.—Monkey 1 (*Cercopithecus patas*): vaccinated.

Monkey 4 (*Cercopithecus patas*): unvaccinated.—This animal runs rather a swinging chart (Fig. 4), the upper limit of which does not, however, rise above 102°·2 F. during the five weeks which preceded the inoculation of the typhus blood. During the incubation period of five days the morning drop in the temperature is eliminated. On the sixth and seventh days the temperature rises a little, and on the eighth and ninth days the febrile reaction is very marked. The fever is maintained through the thirteenth day. The temperature falls on the fourteenth and fifteenth days, and declines very rapidly on the sixteenth and seventeenth days. A very irregular subnormal chart is exhibited during the concluding ten days.

Monkey 7 (*Macacus nemestris*): unvaccinated.—This monkey has an irregular chart (Fig. 5) with a low morning temperature. Between 23rd and 27th March the monkey was subnormal, but was running about his normal temperature at the time of inoculation. The incubation period is about four days; there is a febrile reaction of no great intensity, with a remission of fever from about the tenth day to the thirteenth day; on the fourteenth day the temperature rises again, and after the sixteenth day an irregular temperature is shown until the termination of the chart. The monkey showed signs of being very ill from the twelfth day on; he had attacks of depression and was inclined to sit with his eyes shut; he was very irritable when roused. The cornea of his eyes was unusually brown, and there was a rather marked

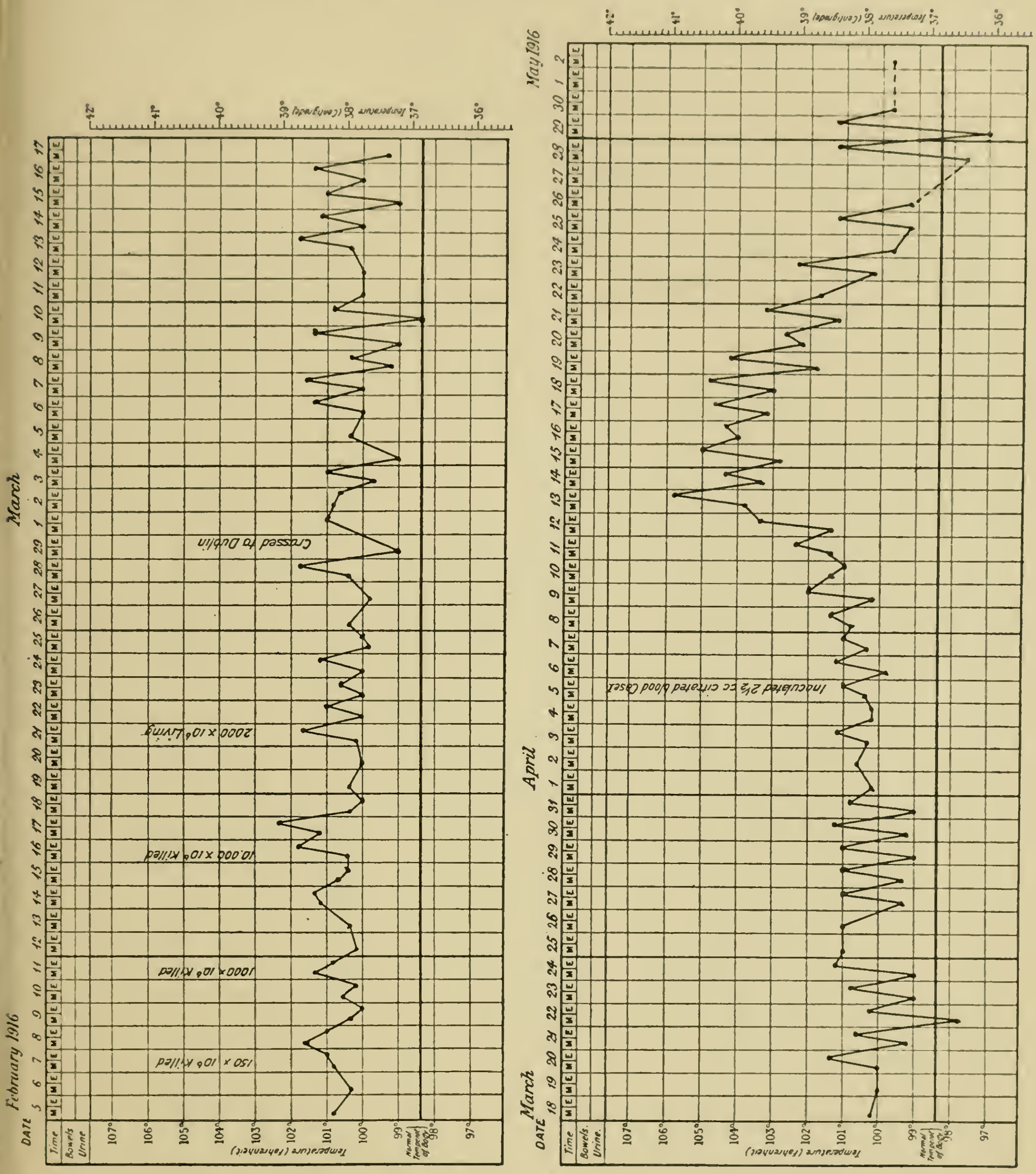

FIG. 2.—**Monkey 8** (*Macacus cynomolgus*): vaccinated.

disinclination to face the light. He ultimately recovered. This is not a very satisfactory chart, the temperature reaction in response to the inoculation of the typhus blood being somewhat irregular in character. There was, however, as far as could be observed, no other morbid condition complicating the

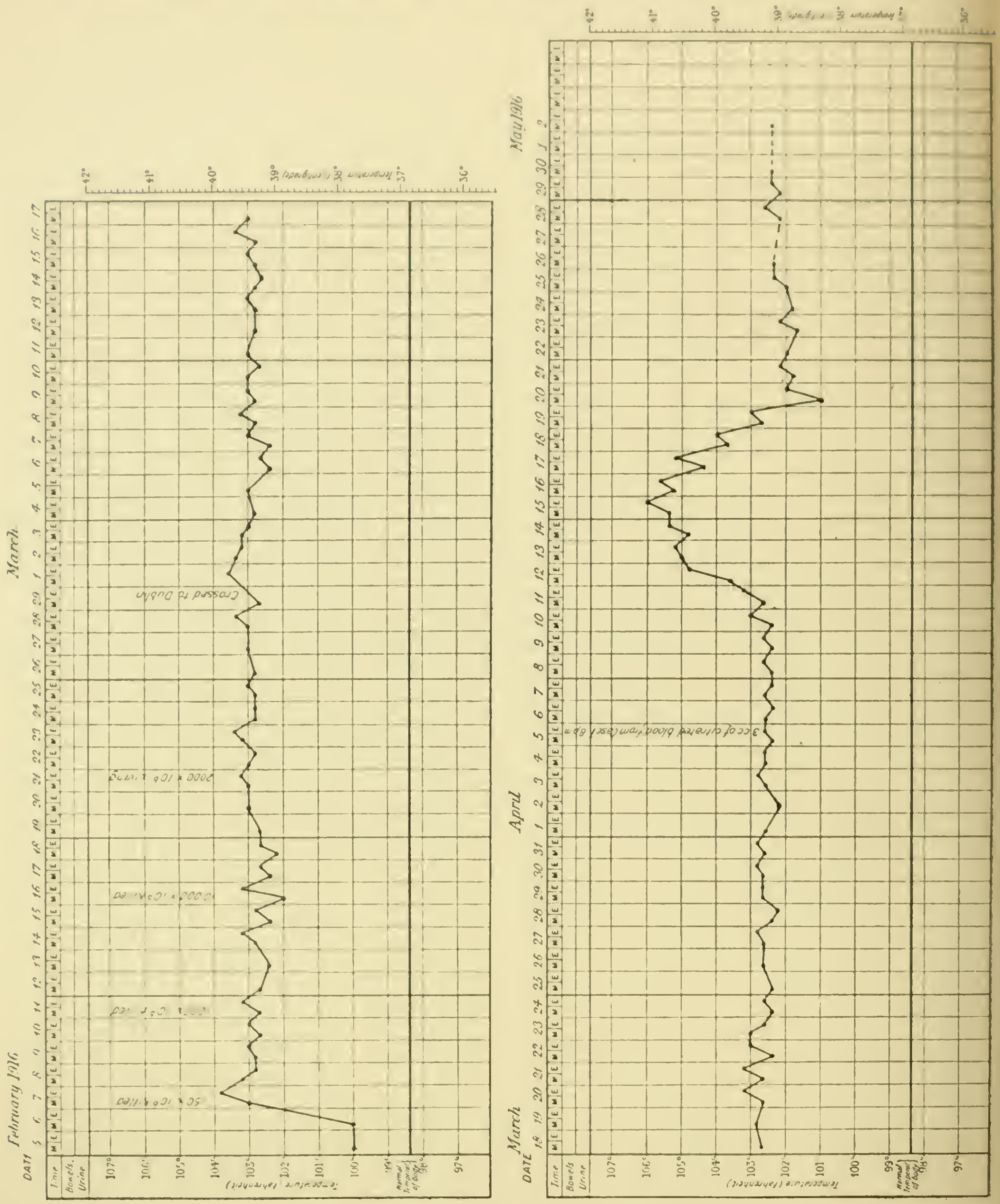

FIG. 3.—Monkey 9 (*Macacus rhesus*): vaccinated.

picture. It must be conceded that, taken by itself, this is not a very convincing animal—there was an undoubted febrile reaction consequent upon the inoculation of the patient's blood, but there is nothing entirely characteristic of typhus in the temperature chart exhibited.

Monkey 21 (*Macacus rhesus*): unvaccinated.—This monkey runs a good chart (Fig. 6), which, however, is rising a little at the time of the inoculation of the patient's blood. There is an incubation period of four days. On the fifth day after the inoculation of the typhus blood there is a rise in temperature, but on the sixth day the temperature is normal; again it rises on the seventh day, and a state of high fever occurs, lasting from the eighth to the thirteenth day. The temperature declines on the fourteenth and fifteenth days, and on the sixteenth day there is a rapid drop to 101°, which is slightly subnormal for this monkey. An irregular rather febrile temperature is shown to the end of the chart. This is quite a good typhus chart from the sixth day on, but it must be noted that the monkey was observed to have enlarged axillary glands on the fourth day after inoculation, and this may have complicated the picture.

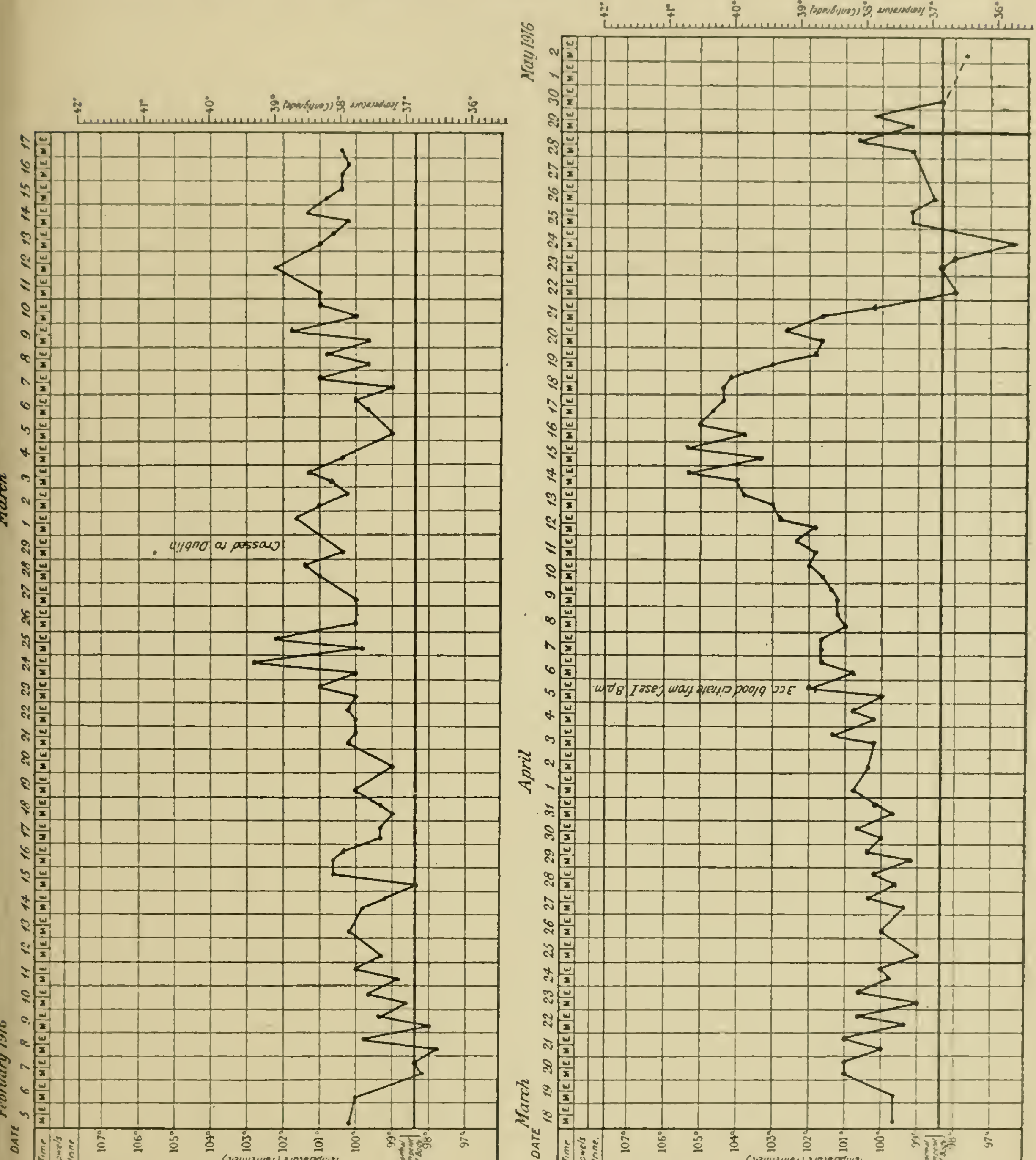

FIG. 4.—Monkey 4 (*Cercopithecus patas*): unvaccinated.

From these six charts it is quite obvious that the vaccination of monkeys 1, 8, and 9, with Dr. Penfold's coccus, had in no way raised their resistance against the febrile attack occasioned by the injection into the peritoneum of blood from a typhus patient. The slight irregularity in the keeping of the charts from 26th April to 2nd May was due to distressful distractions, but it does not seriously interfere with the completeness of the record.

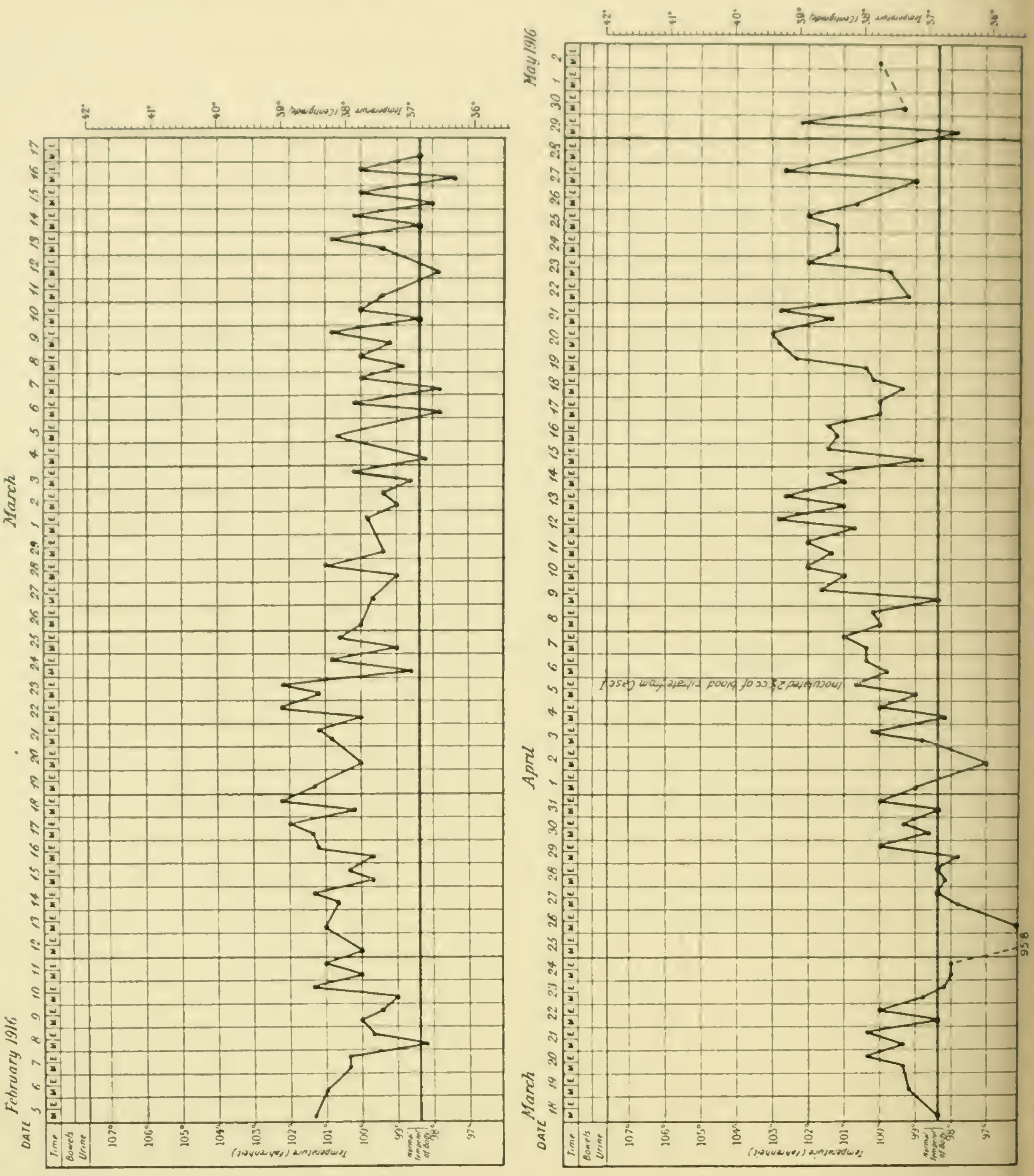

FIG. 5.—**Monkey 7** (*Macacus nemestris*) : unvaccinated.

Bacteriological findings.—0·5 of a cubic centimetre of the citrated blood from the patient was plated after six and a half hours on to three freshly prepared human blood agar plates. These plates contained 2 c.c. of human blood. The method used was to drop the blood citrate mixture derived from the patient from a sterile Pasteur pipette on to the surface of the first blood agar plate and then to spread the material with a sterile glass rod, bent at right angles, proceeding in the usual way to the second and third plates.

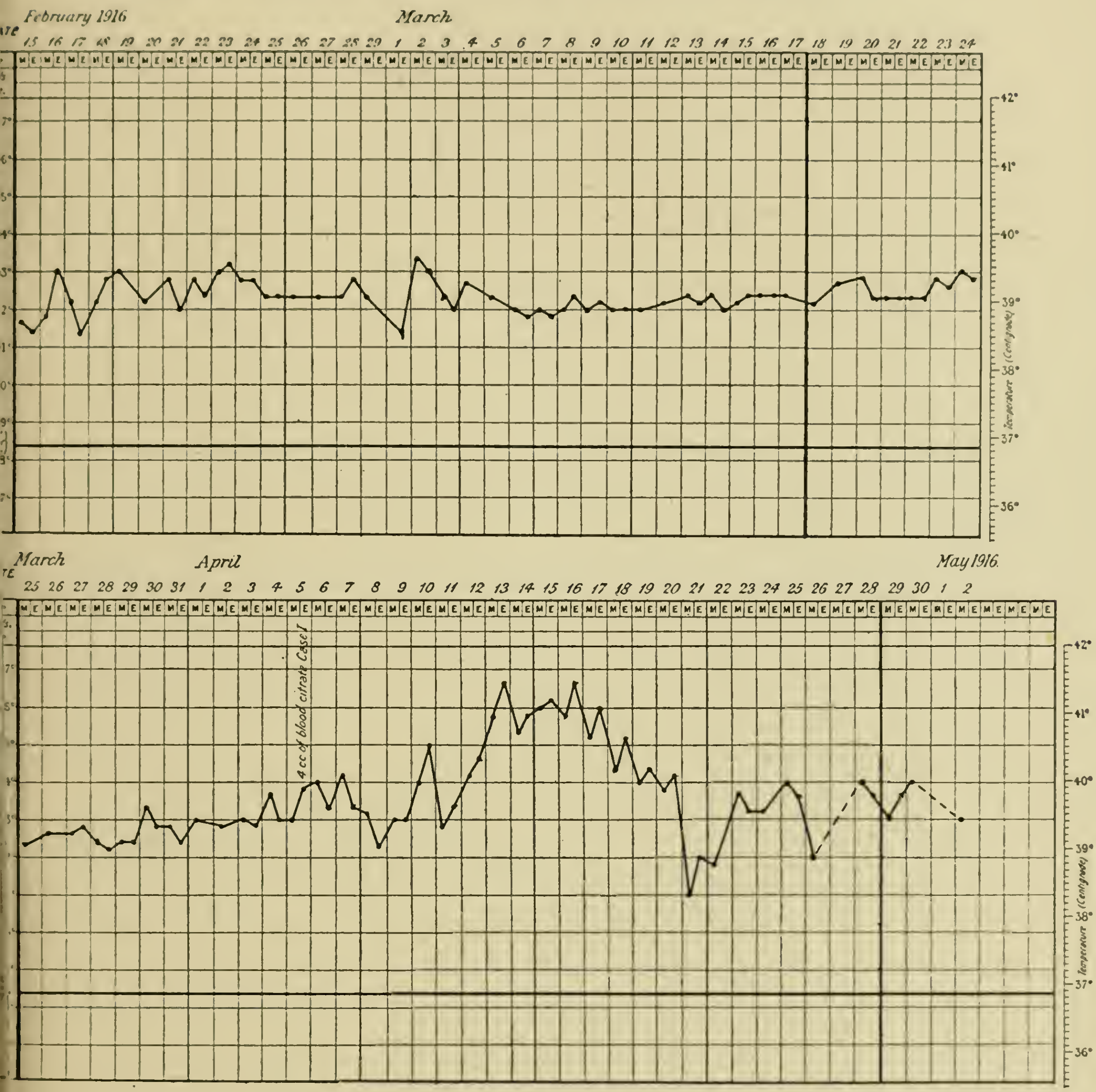

FIG. 6.—**Monkey 21** (*Macacus rhesus*) : unvaccinated.

Upon aerobic incubation, at 37° C., plate 1 showed two white colonies and a minute patch of a greenish-coloured growth at the edge of the plate; this proved to be a Gram-positive coccus growing in pairs and fours. The white colonies were found to consist of a large Gram-negative bacillus which appears to be a contaminating organism from the air. Plate 2 showed no growth of any kind. Plate 3 showed one white colony of the contaminating organism above mentioned, also three colonies of a green colour which produced a green halo upon the blood agar plate; these colonies consisted of a Gram-positive coccus growing in pairs and fours.

This coccus with the green colony appears to be the same organism as that found by Dr. Penfold in his Belfast cases in 1914. It was therefore possible to repeat his results in a case from another outbreak and in a widely removed part of the country. Dr. Penfold's cases were in Belfast in 1914; the present case occurred in the south-west, at Kildysart in County Clare, in 1916.

EXPERIMENT B.

Seven monkeys in all were used; three were vaccinated by the method employed for Experiment A and four were normal monkeys.

Vaccinated monkeys.—No. 13, *Macacus rhesus*; No. 5, *Macacus cynomolgus*; No. 18, *Cebus capucinus.*

Normal monkeys.—Nos. 11, 12, 15, and 22.—*Macacus rhesus.*

The patient, a young woman, took ill, Saturday 8th April. Monday and Tuesday, 9th and 10th April, temperature 102°·8 F. Wednesday, rash appeared, typhus suggested, temperature 99°. Brought into Kildysart hospital, Thursday, 13th, temperature 98°·8; visited Friday, 14th April, temperature 99°.

Blood drawn into sodium citrate solution at 1.45 p.m., patient's temperature 99° at the time. The blood citrate was brought from the hospital at Kildysart to Dublin, and the monkeys inoculated into the peritoneum at 7.50 p.m., that is, six hours after taking the sample. Each monkey had an intraperitoneal injection of 3 c.c. of a mixture of equal parts of citrate and blood.

The monkeys were kept under observation for eighteen days after the inoculation of the patient's blood, but no febrile reaction took place. It was therefore concluded that the case was past the infective period by the time that the sample of blood was taken from the patient. It may be pointed out that although it was the sixth day of the illness, counting from the day on which the patient first considered herself to be ill, the temperature had nevertheless not been above 99° for three days. Against this must, however, be set Nicolle and Blaizot's opinion (1915[1]) that a negative result should not be recorded until forty-five days have elapsed from the time of injecting the typhus blood. The publication of this result had not been noted at the time the experiments were being made. It is, moreover, not quite clear if Nicolle and Blaizot are referring only to subsequent animal passages, notably in the rabbit, or whether they consider that this applies also to an injection into monkeys direct from the patient.

Bacteriological findings.—0·25 c.c. of the blood citrate mixture from the typhus case was plated as before on freshly prepared human blood agar plates, six hours after withdrawal from the patient.

The plates contained 2 c.c. of human blood. Plate 1 showed no growth at all. Plate 2 showed one white colony which proved to be a Gram-positive coccus, and two green colonies with green halos. Plate 3 showed some white colonies and numerous green colonies with green halos. Plate 4 showed no growth. The green colonies were composed of Gram-positive cocci, growing in twos and fours, and were apparently identical with Dr. Penfold's coccus from the Belfast cases.

It is interesting to note that the green coccus was very much more plentiful in this case than in the first, although the latter was more infective for monkeys.

Cultures of the blood from both cases were made in bile salt, but no growth occurred in either case.

Summary and Conclusions.

1. The coccus grown by Dr. Penfold from cases of typhus in Belfast in 1914 was again recovered from the blood in two cases of this disease occurring in the south-west of Ireland in 1916.

2. It was shown in Experiment A that monkeys vaccinated with Dr. Penfold's coccus had acquired no immunity against a subsequent intraperitoneal inoculation of blood from a typhus patient.

3. No evidence was obtained of a causal connection between the coccus found in the blood and the clinical condition known as typhus fever.

Most kind assistance was given by Professor Mettam, Principal of the Royal Veterinary College, Dublin, who contributed the resources of the College to the carrying on of the work. His sympathetic help and that of his staff were of the utmost value, and the success of the experiment was largely due to his generous co-operation. Thanks are due to the Local Government Board of Ireland for their kindness in notifying the cases. I am also indebted to Drs. Garry and Brennan for their courtesy in allowing their patients to be visited.

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A CONTRIBUTION TO THE STUDY OF AMBOCEPTORS AND RECEPTORS.

SECOND COMMUNICATION ON HETEROLOGOUS IMMUNITY TO MALIGNANT MOUSE TUMOURS.

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SINCE Forssman (1911) discovered a heterologous haemolysin obtained by immunising rabbit against guinea-pig organs—kidney, liver, etc.—and having the property of dissolving sheep blood corpuscles but not those of the guinea-pig, many authors have devoted their time to this subject, and to the elucidation of the relationship between antigen and antibody.

Thus, Orudschiew (1913) succeeded in obtaining haemolytic amboceptors by immunising with guinea-pig serum, while Spaet (1914) claimed that the leucocytes of this animal can also evoke the production of the same amboceptors. In 1913 Morgenroth demonstrated two haemolysins obtained by immunising rabbit against mouse kidney and against mouse carcinoma, and noted that it was a difficult task to decide whether the haemolysins were identical or partially different. In a later paper, Morgenroth and Bieling (1915) published a detailed account dealing with this subject. For this purpose they employed the methods of fixation and transgression, and declared that the haemolysins were not identical, but differed from one another to a certain extent.

In a previous paper (1915) I investigated the serological reactions of malignant mouse and rat tumour antisera, *i.e.* haemolysis, haemagglutination, complement fixation, precipitin reaction and cytolysis. My experiments were all made in cases of heterologous immunity and working along bacteriological lines, since in the case of homologous immunity to mouse tumours (*e.g.* immunising mouse against mouse tumours and certain normal tissues—for example mouse embryo-skin) no specific antibody has yet been found.

In the present paper the investigations concern the relationship between mouse tumour and guinea-pig kidney haemolysins and goat-erythrocyte amboceptors respectively, according to the methods of Morgenroth and Bieling.

I. Immunising Methods.

To immunise with organic cells and obtain heterologous haemolysin many authors have applied the methods, in some cases modified, already used by Forssman. The methods used by Morgenroth and Bieling are very well adapted for the obtaining of exact results. There are, however, in spite of everything, some unavoidable difficulties when immunising with organic and tumour cells, because, apart from the difficulty that exists in obtaining the antigen absolutely free from blood, it is scarcely possible to separate the connective tissue from the parenchyma; an example of this is seen in Bail and Margulies's (1913) use of guinea-pig kidney as antigen.

However, one can probably neglect the influence of connective tissue, because in mouse tumour and guinea-pig kidney the parenchyma is so largely in excess.

Morgenroth and Bieling's methods were therefore adopted and are as follows:

The immunising materials—mouse tumour and guinea-pig kidney—are removed from animals which have been bled out and are cut up under sterile conditions and then washed with normal saline in order to get rid of as much blood as possible. These materials are ground down in a sterile mortar without any addition of sea sand. To the pulp a certain quantity of saline is added and the whole filtered through an ordinary handkerchief under slight pressure. The emulsions thus prepared were injected intraperitoneally into rabbits, twice or three times with intervals of from eight to ten days.

As to the dosage, Forssman had already suggested that a large quantity does not give a proportionately high immunisation, and Morgenroth and Bieling confirming this assertion, used for each injection emulsion corresponding to 1–2 grms. of original material.

Eight days after the last inoculation, the animals were bled from the carotid under narcosis. The immunised sera obtained were inactivated the next day at 56° C. for half-an-hour. To test the haemolytic reaction 0·5 c.c. of a 5% washed goat erythrocyte suspension was employed The complement was 1 c.c. of a 1 in 10 dilution of fresh guinea-pig serum, the absolute quantity of complement in each tube thus being 0·1 c.c. The minimal dose which still can cause complete haemolysis under these conditions was named the amboceptor unit.

The degrees of haemolysis were distinguished as follows:

c. = complete
n.c. = nearly complete
m. = marked
sl. = slight
tr. = trace
n. = none

The following protocols show the haemolysin antigenic power of guinea-pig kidney and mouse tumour.

Immunisation with guinea-pig kidney.

Rabbit 1.

1915. 8th Nov. 5·0 c.c. of 20% guinea-pig kidney emulsion intraperitoneally.
15th ,, 5·0 ,, ,, ,, ,,
22nd ,, 10·0 ,, ,, ,, ,,
30th ,, Bled out.
1st Dec. Amboceptor unit = 0·005.

Rabbit 2.

1915. 8th Nov. 5·0 c.c. of 20% guinea-pig kidney emulsion intraperitoneally.
15th ,, 5·0 ,, ,, ,, ,,
22nd , 10·0 ,, ,, ,, ,,
30th ,, Bled out.
1st Dec. Amboceptor unit = 0·002.

Rabbit 3.

1915. 8th Nov. 5·0 c.c. of 20% guinea-pig kidney emulsion intraperitoneally.
15th ,, 5·0 ,, ,, ,, ,,
22nd ,, 10·0 ,, ,, ,, ,,
30th ,, Bled out.
1st Dec. Amboceptor unit = 0·001.

Immunisation with mouse tumour.

For this purpose mouse carcinoma 199 and sarcoma 37 p. which were supplied by the kindness of Dr Murray, the Director of the Imperial Cancer Research Fund, London, were used. The virulence of these tumours is so great that their takes by transplantation are practically 100 per cent., and their proliferative activity so rapid that they reach from 1 grm. to 1·5 grms. two weeks after an inoculation of 0·03 c.c.

Immunisation with mouse carcinoma 199.

Rabbit 4.

1915.	19th	Nov.	7·0 c.c. of 25 % mouse carcinoma 199 emulsion intraperitoneally.
	30th	,,	6·0 ,, ,, ,, ,,
	10th	Dec.	10·0 ,, ,, ,, ,,
	18th	,,	Bled out.
	19th	,,	Amboceptor unit = 0·005 c.c.

Rabbit 5.

1915.	19th	Nov.	5·0 c.c. of 25 % mouse carcinoma 199 emulsion intraperitoneally.
	30th	,,	6·0 ,, ,, ,, ,,
	10th	Dec.	10·0 ,, ,, ,, ,,
	18th	,,	Bled out.
	19th	,,	Amboceptor unit = 0·006 c.c.

Rabbit 6.

1915.	19th	Nov.	5·0 c.c. of 25 % mouse carcinoma 199 emulsion intraperitoneally.
	30th	,,	6·0 ,, ,, ,, ,,
	10th	Dec.	10·0 ,, ,, ,, ,,
	15th	,,	Died.

Immunisation with mouse sarcoma 37 p.

Rabbit 7.

1915.	2nd	Dec.	10·0 c.c. of 25 % mouse sarcoma 37 p. emulsion intraperitoneally.
	10th	,,	7·0 ,, ,, ,, ,,
	20th	,,	10·0 ,, ,, ,, ,,
	28th	,,	Bled out.
	29th	,,	Amboceptor unit = 0·02 c.c.

Rabbit 8.

1915.	2nd	Dec.	10·0 c.c. of 25 % mouse sarcoma 37 p. emulsion intraperitoneally.
	10th	,,	8·0 ,, ,, ,, ,,
	18th	,,	Bled out.
	19th	,,	Amboceptor unit = 0·025 c.c.

According to Morgenroth and Bieling, mouse tumour—carcinoma—contains more antigenic receptors than mouse kidney, but from the above experiments, although few in number, the antigenic power of mouse tumour, either carcinoma or sarcoma, does not appear to exceed that of guinea-pig kidney.

II. Fixation Experiments.

The guinea-pig kidney and mouse tumour emulsions employed in the following experiments were 10 per cent. by weight, and prepared according to Morgenroth and Bieling; that is to say, the materials were removed from animals, which had been bled out, were cut up, washed with normal saline in order to free them from blood as much as possible, and then centrifuged. Thus the organ and tumour cells were separated from the blood corpuscles, 10 per cent. suspensions of the precipitated cells being made by weight with normal saline. I found 2·0 c.c. of these suspensions an adequate amount for fixation with the serum used.

1. Fixation experiments with mouse carcinoma 199 serum. Rabbit No. 4. A.U. = 0·005 c.c.

For control was used, as in Morgenroth and Bieling's experiments, rabbit kidney emulsion prepared in the way already described.

To 1·0 c.c. (200 A.U.) of the inactivated carcinoma serum was added 2·0 c.c. of 10 % carcinoma, guinea-pig and rabbit kidney emulsions respectively, thus making 3·0 c.c. in all:

1·0 c.c. carcinoma serum (200 A.U.) + 2·0 c.c. of 10 % carcinoma emulsion.
1·0 ,, ,, ,, ,, + 2·0 ,, of 10 % guinea-pig kidney emulsion.
1·0 ,, ,, ,, ,, + 2·0 ,, of 10 % rabbit kidney emulsion.

The mixtures were placed for one hour at 37° C. and afterwards centrifuged. The slightly opaque supernatant fluids were then tested haemolytically, using 0·1 c.c. guinea-pig complement and 0·5 c.c. of 5 % goat erythrocyte suspension. After placing for one hour and a half at 37° C., the tubes were kept in the cold room and the result observed the following morning.

The following table shows the results:

TABLE I.

Mouse carcinoma serum.

	Absorbed serum used in haemolytic test	Haemolytic strength before absorption	Haemolysis after absorption with		
			Mouse carcinoma	Guinea-pig kidney	Rabbit kidney
1.	1·2 c.c.	80 A.U.	c.	c.	c.
2.	0·6 ,,	40 ,,	n.c.	,,	,,
3.	0·3 ,,	20 ,,	n.c.—m.	n.c.	,,
4.	0·15 ,,	10 ,,	m.	m.	,,
5.	0·075 ,,	5 ,,	sl.	sl.	,,
6.	0·03 ,,	2 ,,	sl.—tr.	tr.	,,
7.	0·015 ,,	1 ,,	tr.—n.	tr.—n.	n.c.
8.	0·01 ,,	$\frac{2}{3}$,,	n	n.	m

Mouse carcinoma is thus seen to fix very strongly, while guinea-pig kidney was rather feebler, and by calculating according to Morgenroth and Bieling's method, it is found that mouse carcinoma fixed from at least $\frac{39}{40}$ to $\frac{79}{80}$ of the amboceptors, namely, from 195 to 197 A.U., while guinea-pig kidney deviated $\frac{19}{20}$ to $\frac{39}{40}$; namely, from 190 to 195 A.U. The attempt to fix with rabbit kidney, as Morgenroth has already noted, does not succeed, so that 1 amboceptor unit after absorption still caused practically complete haemolysis. Thus it is clear that rabbit kidney does not fix haemolytic amboceptors, in the same way as, according to Forssman, it does not elicit the antibodies.

2. Fixation experiments with guinea-pig kidney serum. Rabbit No. 3. A.U. = 0·001 c.c.

To tubes containing 0·2 c.c. (200 A.U.) of the serum and 0·8 c.c. saline were added 2·0 c.c. of guinea-pig, mouse carcinoma and rabbit kidney emulsions respectively, the total amount thus being 3 c.c.

0·2 c.c. guinea-pig kidney serum (200 A.U.) + 0·8 c.c. saline + 2·0 c.c. guinea-pig kidney emulsion.

0·2 c.c. guinea-pig kidney serum (200 A.U.) + 0·8 c.c. saline + 2·0 c.c. mouse carcinoma emulsion.

0·2 c.c. guinea-pig kidney serum (200 A.U.) + 0·8 c.c. saline + 2·0 c.c. rabbit kidney emulsion.

After treating the mixtures as before, the supernatant fluids were obtained and tested for haemolytic reaction.

TABLE II.

Guinea-pig kidney serum.

	Absorbed serum used in haemolytic test	Haemolytic strength before absorption	Haemolysis after absorption with		
			Guinea-pig kidney	Mouse carcinoma	Rabbit kidney
1.	1·2 c.c.	80 A.U.	c.	c.	c.
2.	0·6 ,,	40 ,,	n.c.	,,	,,
3.	0·3 ,,	20 ,,	m.	,,	,,
4.	0·15 ,,	10 ,,	sl.	,,	,,
5.	0·075 ,,	5 ,,	tr.	n.c.	,,
6.	0·03 ,,	2 ,,	n.	m.	,,
7.	0·015 ,,	1 ,,	,,	tr.	n.c.
8.	0·01 ,,	$\frac{2}{3}$,,	,,	n.	m.

The fixing power of the guinea-pig kidney emulsion for the amboceptors of the homologous serum lies between $\frac{79}{80}$ and $\frac{39}{40}$, whilst that of the carcinoma emulsion lies between $\frac{9}{10}$ and $\frac{4}{5}$; in other words, although the carcinoma antigens were capable of fixing haemolytic antibodies, these were most strongly fixed by homologous antigens. Rabbit kidney again showed no power to absorb the haemolysin, so that one amboceptor unit, after treatment, still caused nearly complete haemolysis. However, the behaviour of goat haemolysin, with mouse carcinoma and guinea-pig kidney emulsion, is quite different.

3. Fixation experiments with goat haemolysin. A.U. = 0·0005 c.c. The mixture this time consisted of 0·1 c.c. (200 A.U.) of the goat haemolysin, 0·9 c.c. saline and 2·0 c.c. of goat erythrocytes (10 % by weight), guinea-pig and rabbit kidney suspensions respectively. After treating the mixtures as before, the haemolytic power of the supernatant fluids was tested.

TABLE III.

Goat red cell serum.

	Absorbed serum used in haemolytic test	Haemolytic strength before absorption	Haemolysis after absorption with			
			Goat erythrocytes	Mouse carcinoma	Guinea-pig kidney	Rabbit kidney
1.	0·6 c.c.	40 A.U.	c.	c.	c.	c.
2.	0·3 ,,	20 ,,	,,	,,	,,	,,
3.	0·15 ,,	10 ,,	m.	,,	,,	,,
4.	0·075 ,,	5 ,,	sl.	,,	,,	,,
5.	0·03 ,,	2 ,,	n.	,,	,,	,,
6.	0·015 ,,	1 ,,	,,	n.c.	,,	,,
7.	0·0075 ,,	½ ,,	,,	m.	m.	n.c.
8.	0·0004 ,,	¼ ,,	,,	tr.	tr.	sl.

As the table demonstrates, goat blood cells fix the amboceptors well, while not only rabbit kidney, but carcinoma and guinea-pig kidney do not absorb them practically at all. This result is very curious, because both mouse carcinoma and guinea-pig kidney contain antigen for the production of amboceptors haemolytic to goat red cells. To explain this fact, the following statement is probably admissible: goat erythrocytes contain many groups of receptors able to produce amboceptors haemolytic to goat blood cells, while mouse carcinoma and guinea-pig kidney contain some of these groups only.

If, therefore, haemolytic serum produced by immunisation with goat blood cells is absorbed with mouse carcinoma or guinea-pig kidney emulsions, those amboceptor groups corresponding to their own receptors are fixed, while there still remain the other groups of antibodies to cause haemolysis. It is certainly due to a want of any receptors in common that rabbit kidney did not fix goat haemolysin amboceptors.

Another question approached in this paper is concerned with the fixing power of goat blood corpuscles for the haemolytic amboceptors of mouse carcinoma and guinea-pig kidney serum. Although Morgenroth and Bieling observed that goat erythrocytes fix the haemolytic antibodies of mouse carcinoma serum more strongly than mouse kidney emulsion, they did not publish an exact account.

4. Fixation experiments with goat blood cells.

To 1·0 c.c. (200 A.U.) of mouse carcinoma serum and 0·2 c.c. (200 A.U.) of guinea-pig kidney, plus 0·8 c.c. saline, 2·0 c.c. of 10 % goat erythrocytes were added, while the same amount of mouse carcinoma and guinea-pig kidney emulsion was added, each to its corresponding serum as control.

1·0 c.c. mouse carcinoma serum (200 A.U.) + 2·0 c.c. of 10 % goat blood cells.
1·0 ,, ,, ,, ,, + 2·0 ,, of 10 % mouse carcinoma emulsion.
0·2 ,, guinea-pig kidney serum (200 A.U.) + 0·8 c.c. saline + 2·0 c.c. of 10 % goat blood cells.
0·2 c.c. guinea-pig kidney serum (200 A.U.) + 0·8 c.c. saline + 2·0 c.c. of 10 % guinea-pig kidney emulsion.

The mixtures were treated as before and the haemolytic reaction of the supernatant fluids tested.

TABLE IV. A.

Mouse carcinoma serum.

	Absorbed serum used in haemolytic test	Haemolytic strength before absorption	Haemolysis after absorption with	
			Goat erythrocytes	Mouse carcinoma (control)
1.	1·2 c.c.	80 A.U.	m.	c.
2.	0·6 ,,	40 ,,	sl.	n.c.
3.	0·3 ,,	20 ,,	tr.	n.c.—m.
4.	0·15 ,,	10 ,,	,,	m.
5.	0·075 ,,	5 ,,	n.	sl.
6.	0·03 ,,	2 ,,	,,	sl.—tr.
7.	0·015 ,,	1 ,,	,,	tr.—n.
8.	0·01 ,,	$\frac{2}{3}$,,	,,	n.

TABLE IV. B.

Guinea-pig kidney serum.

	Absorbed serum used in haemolytic test	Haemolytic strength before absorption	Haemolysis after absorption with	
			Goat erythrocytes	Guinea-pig kidney (control)
1.	1·2 c.c.	80 A.U.	n.c.	c.
2.	0·6 ,,	40 ,,	m.	n.c.
3.	0·3 ,,	20 ,	sl.	m.
4.	0·15 ,,	10 ,,	tr.	sl.
5.	0·075 ,,	5 ,,	n.	tr.
6.	0·03 ,,	2 ,,	,,	n.
7.	0·015 ,,	1 ,,	,,	,,
8.	0·01 ,,	$\frac{2}{3}$,,	,,	,,

As the preceding experiments demonstrate, goat blood cells have a much greater fixing power for both haemolytic amboceptors than their homologous antigens, this being particularly the case with the carcinoma amboceptors.

It has been already stated that rabbit kidney fixes the amboceptors of neither mouse carcinoma, guinea-pig kidney, nor goat erythrocyte antiserum, and the immunising of rabbit with rabbit kidney cells conversely does not elicit the production of haemolysins. Consequently, it is natural to suppose that the organs of other animals, for example rat kidney which, according to Forssman and Morgenroth, does not contain the haemolysin antigen, cannot have any fixing power for the amboceptors of mouse carcinoma, guinea-pig kidney and goat erythrocyte antisera.

5. Fixation experiment with rat kidney emulsion.

The experiment was performed as follows:

A. 0·5 c.c. (100 A.U.) mouse carcinoma serum + 0·5 c.c. saline + 2·0 c.c. rat kidney emulsion.

B. 0·1 c.c. (100 A.U.) guinea-pig kidney serum + 0·9 c.c. saline + 2·0 c.c. rat kidney emulsion.

C. 0·1 c.c. (200 A.U.) goat haemolysin + 0·9 c.c. saline + 2·0 c.c. rat kidney emulsion.

These mixtures were treated as before and the haemolytic reaction of the supernatant fluids tested.

TABLE V. A.

Mouse carcinoma serum.

	Absorbed serum used in haemolytic test	Haemolytic strength before absorption	Haemolysis after absorption with	
			Mouse carcinoma	Rat kidney
1.	1·2 c.c.	40 A.U.	c.	c.
2.	0·6 ,,	20 ,,	n.c.	,,
3.	0·3 ,,	10 ,,	,,	,,
4.	0·15 ,,	5 ,,	m.	,,
5.	0·075 ,,	2½ ,,	sl.	,,
6.	0·03 ,,	1 ,,	sl.—tr.	,,
7.	0·015 ,,	½ ,,	n.	n.c.
8.	0·01 ,,	⅓ ,,	,,	sl.

TABLE V. B.

Guinea-pig kidney serum.

	Absorbed serum used in haemolytic test	Haemolytic strength before absorption	Haemolysis after absorption with	
			Guinea-pig kidney	Rat kidney
1.	1·2 c.c.	40 A.U.	c.	c.
2.	0·6 ,,	20 ,,	n.c.	,,
3.	0·3 ,,	10 ,,	m.	,,
4.	0·15 ,,	5 ,,	sl.	,,
5.	0·075 ,,	2½ ,,	tr.	,,
6.	0·03 ,,	1 ,,	n.	,,
7.	0·015 ,,	½ ,,	,,	n.c.
8	0·01 ,,	⅓ ,,	,,	m.

TABLE V. C.

Goat erythrocyte serum.

	Absorbed serum used in haemolytic test	Haemolytic strength before absorption	Haemolysis after absorption with	
			Goat erythrocytes	Rat kidney
1.	0·6 ,,	40 A.U.	c.	c.
2.	0·3 ,,	20 ,,	,,	,,
3.	0·15 ,,	10 ,,	m.	,,
4.	0·075 ,,	5 ,,	sl.	,,
5.	0·03 ,,	2 ,,	n.	,,
6.	0·015 ,,	1 ,,	,,	,,
7.	0·0075 ,,	½ ,,	,,	m.
8.	0·004 ,,	¼ ,,	,,	sl.

The tables show that the supposition was right, that is to say, rat kidney emulsion does not fix the amboceptors practically at all; so that one amboceptor unit in all cases still caused after treatment nearly complete haemolysis. So, it is confirmed that, as Morgenroth asserted, the fixation reaction is specific.

The mouse tumour used in the foregoing experiments has throughout been carcinoma.

It still remained to be seen whether mouse sarcoma serum would behave in the same way. For this purpose serum 37 p. was used, and exactly the same method applied as before.

6. Fixation experiment. Amboceptor unit of serum 37 p.=0·02 c.c.

It is regrettable that this time I could not for certain reasons use homologous tumour emulsion; mouse carcinoma emulsion had to be employed instead.

A. 2·0 c.c. (100 A.U.) of the serum + 2·0 c.c. mouse carcinoma emulsion.

B. 2·0 c.c. (100 A.U.) of the serum + 2·0 c.c. guinea-pig kidney emulsion.

Here the mixtures amounted to 4·0 c.c., it being necessary to take 2·0 c.c. of the serum in order to obtain 100 amboceptor units.

After absorption the haemolytic reaction was carried out on the supernatant fluids.

TABLE VI.

Mouse sarcoma serum.

	Absorbed serum used in haemolytic test	Haemolytic strength before absorption	Haemolysis after absorption with	
			Mouse carcinoma	Guinea-pig kidney
1	0·8 c.c.	20 A.U.	c.	m.
2.	0·4 ,,	10 ,,	n.c.	,,
3.	0·2 ,,	5 ,,	m.	sl.
4.	0·1 ,,	2½ ,,	m.—sl.	tr.
5.	0·04 ,,	1 ,,	sl.	n.
6.	0·02 ,,	½ ,,	tr.	,,

It is seen that guinea-pig kidney fixes the amboceptors of mouse sarcoma serum better than mouse carcinoma. In other words, it seems that the former contains more lysinogen than the latter for the amboceptors of sarcoma 37 p. antiserum.

III. TRANSGRESSION EXPERIMENTS.

The cell-sediments produced by centrifugation (either mouse tumour or guinea-pig kidney) were carefully separated from their supernatant fluids and 10 % suspensions prepared each time before the experiment. The only difference was that the quantity of suspension consisted of 2·0 c.c. while Morgenroth and Bieling used 2·5 c.c.

1. Transgression experiment with mouse carcinoma serum.

Here the sediment obtained in fixation experiment 1, whose protocol is produced here, was used.

1·0 c.c. (200 A.U.) mouse carcinoma serum + 2·0 c.c. carcinoma 199 emulsion = 3·0 c.c.
1·0 c.c. (200 A.U.) mouse carcinoma serum + 2·0 c.c. guinea-pig kidney emulsion = 3·0 c.c.

To the sediments produced by centrifugation was added saline to the original volume (3·0 c.c.). With diminishing amounts of these cell suspensions 0·5 c.c. of 5 % goat erythrocytes was mixed; after incubating at 37° C. for one hour, 0·1 c.c. complement was added, and the tubes again placed in the hot room for two hours.

The following table shows the results:

TABLE VII.

		Haemolysis after transgression with	
	Amount of cell suspension	Mouse carcinoma	Guinea pig kidney
1.	1·0 c.c.	sl.—m.	n.c.
2.	0·5 ,,	tr.	m.
3.	0·3 ,,	n.	,,
4.	0·2 ,,	,,	sl.
5.	0·1 ,,	,,	tr.
6.	0·05 ,,	,,	n.

Both suspensions, but especially carcinoma emulsion retained a considerable quantity of the fixed amboceptors and gave to the goat blood cells some of them only. In other words, carcinoma cells fixed amboceptors from carcinoma serum more permanently than guinea-pig kidney did.

2. Transgression experiment with guinea-pig kidney serum.

Here the cell sediments obtained in fixation experiment 2 as shown in the following protocol were used:

0·2 c.c. (200 A.U.) guinea-pig kidney serum + 2·0 c.c. guinea-pig kidney emulsion + 0·8 c.c. saline = 3·0 c.c.

0·2 c.c. (200 A.U.) guinea-pig kidney serum + 2·0 c.c. mouse carcinoma 199 emulsion + 0·8 c.c. saline = 3·0 c.c.

To the sediments was added saline to the original volume (3·0 c.c.). The haemolytic reaction after the transgression was tested in the same way as before.

TABLE VIII.

		Haemolysis after transgression with	
	Amount of cell suspension	Guinea-pig kidney	Mouse carcinoma
1.	1·0 c.c.	c.	c.
2.	0·5 ,,	,,	,,
3.	0·3 ,,	,,	,,
4.	0·2 ,,	m.	,,
5.	0·1 ,,	sl.	n.c.
6.	0·05 ,,	tr.	m.

The amount of guinea-pig kidney suspension which caused complete haemolysis is 0·3 c.c., while that of carcinoma suspension is 0·2 c.c. Thus it is seen that the kidney cells have bound the amboceptors of the kidney serum more strongly than the carcinoma cells.

3. Transgression experiment with mouse sarcoma serum.

The sediments used were those obtained in fixation experiment 6, the following being the protocol:

2·0 c.c. (100 A.U.) mouse sarcoma serum 37 p. + 2·0 c.c. mouse carcinoma 199 emulsion = 4·0 c.c.

2·0 c.c. (100 A.U.) mouse sarcoma serum 37 p. + 2·0 c.c. guinea-pig kidney emulsion = 4·0 c.c.

The haemolytic reaction after transgression was carried out as before.

TABLE IX.

		Haemolysis after transgression with	
	Amount of cell suspension	Mouse carcinoma	Guinea-pig kidney
1.	1·0 c.c.	n.c.	n.c.
2.	0·5 ,,	m.—sl.	m.
3.	0·3 ,,	sl.	sl.
4.	0·2 ,,	tr.	tr.
5.	0·1 ,,	n.	n.
6.	0·05 ,,	n.	n.

Mouse carcinoma cells and kidney cells bound the amboceptors of mouse sarcoma serum 37 p. strongly and practically to the same degree —1·0 c.c. of the suspensions not causing complete haemolysis.

CONCLUSIONS.

1. Mouse carcinoma cells fix the haemolytic amboceptors of homologous serum more strongly than guinea-pig kidney cells.

2. Guinea-pig kidney cells fix the amboceptors of guinea-pig kidney antiserum more strongly than mouse carcinoma cells.

3. Goat blood cells are capable of fixing the amboceptors of both sera particularly well.

4. However, while goat blood cells can naturally absorb goat haemolysin, neither carcinoma nor kidney cells have any power of fixing goat haemolysin practically at all.

5. Rabbit and rat kidney cells, as they do not contain the lysinogen, do not absorb haemolytic amboceptors from the three antisera.

6. This fixation reaction is therefore to be regarded as specific.

7. Transgression experiments show that homologous antigens bind their amboceptors more strongly than heterologous; thus in all probability there is no complete identity between the haemolytic amboceptors which are produced by immunising rabbit against mouse tumours—carcinoma and sarcoma—and guinea-pig kidney. It will be remembered that Morgenroth and Bieling could not establish identity between carcinoma and mouse kidney haemolysis.

In conclusion, I desire to express my thanks to Dr Schütze for his advice in the course of this work.

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A CONTRIBUTION TO THE STUDY OF AMBOCEPTORS AND RECEPTORS.

THIRD COMMUNICATION ON HETEROLOGOUS IMMUNITY TO MALIGNANT MOUSE TUMOURS.

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It must be due to an incidental distribution of receptors that guinea-pig organs and mouse tumours are able to elicit haemolysins for goat erythrocytes, when they are inoculated into rabbit, because antigens are generally found in common only in closely related substances. So, if this relation exists between them, there arises a further question, namely, whether they may not be able to evolve other mutual antibodies; in other words, to what degree they may have antigenic substances in common. I have already noted (1915) that mouse tumour antisera contain complement fixing antibodies, using extracts of mouse and alien tumours as antigens. Michaelis and Fleischmann (1906) reported that rabbit sera immunised against guinea-pig and mouse liver fixed complement in the presence of their homologous antigens, namely, extracts of the livers of guinea-pig and mouse. Later, Fleischmann and Davidsohn (1908) published an extended investigation upon the same subject. So, it is easy to suppose that mouse tumour, guinea-pig kidney and goat erythrocyte antisera contain complement fixing antibodies. Haemagglutination, also, enters into the question, because the sera mentioned above are all haemolytic for goat erythrocytes.

I. Complement Fixation.

The immune sera employed for these experiments are those which were used for a previous work, namely:

1. Mouse carcinoma 199 antiserum,
2. Mouse sarcoma 37 p. antiserum,
3. Guinea-pig kidney antiserum, and
4. Goat erythrocyte antiserum.

All are naturally haemolytic to goat blood cells, and in order to carry out the complement fixation reaction with these sera, it was necessary to eliminate the haemolytic amboceptors from them (goat erythrocytes being used in the haemolytic system), this being done as follows:

In the case of mouse tumour and guinea-pig kidney sera, goat red cells were added in the proportion of 0·5 c.c. goat red cell deposit obtained by washing with saline, to 1·0 c.c. of serum, but in the case of goat erythrocyte serum where this quantity was not sufficient (A.U. = 0·001) it was necessary to absorb twice with 1·0 c.c. of goat blood cells to 1·0 c.c. of the serum. For antigen, watery extracts of mouse carcinoma and guinea-pig kidney, and alcoholic extract of goat blood corpuscles were employed. To prepare alcoholic goat erythrocyte extract I used v. Dungern's method (1912).

The author made up 20 % alcoholic extract from human blood corpuscles in order to test complement fixation with cancer patient serum. As the amount to be used he suggested 0·4 c.c. of this extract after a dilution of 1 in 2 with normal saline. In the present case the same amount of alcoholic goat erythrocyte extract has been employed. Von Dungern's method is, however, not suitable for the preparation of mouse tumour extracts, owing to precipitates being produced in some quantity when dilution with saline takes place. Consequently, watery extracts were employed. As to the method of preparation, healthy tumours were removed from animals which had been bled out, were cut up and washed with normal saline, and 10 % emulsions by weight of the tumour cells were made in physiological saline. These emulsions, after being kept in the cold room for two days, were centrifuged and 0·4 c.c. of the supernatant fluid employed for the reaction—double the quantity having been found not to interfere with complete haemolysis. With guinea-pig kidney extract the same method exactly was applied, and the same quantity employed. In the haemolytic system, 0·5 c.c. of 5 % goat erythrocyte suspension prepared from the deposit was used. The complement dose was 0·5 c.c. of a 1 in 10 dilution of normal fresh guinea-pig serum.

The results were noted after the tubes had been at a temperature of 37° C. for two hours, the degrees of haemolysis being distinguished as follows:

c. = complete
n.c. = nearly complete
m. = marked
sl. = slight
tr. = trace
n. = none

As control, it is necessary to test for complement fixation with normal rabbit serum, using the extracts already mentioned as antigens. For this purpose, sera from several normal rabbits were taken and examined after treating with goat blood cells just as in the cases of mouse tumour and guinea-pig kidney antisera. The results obtained from three of the rabbits are those seen in Table I.

TABLE I.

Complement fixation by normal rabbit serum with mouse carcinoma, guinea-pig kidney and goat erythrocyte extracts.

	Inactivated normal rabbit serum	Mouse carcinoma extract 0·4 c.c.	Guinea-pig kidney extract 0·4 c.c.	Goat erythrocyte extract 0·4 c.c.
1.	0·1 c.c.	c.	c.	c.
2.	0·05 ,,	,,	,,	,,
3.	0·025 ,,	,,	,,	,,
4.	0·01 ,,	,,	,,	,,

Two further rabbits, however, gave different results, being those shown in Table II.

TABLE II.

Complement fixation by normal rabbit serum with mouse carcinoma, guinea-pig kidney and goat erythrocyte extracts.

	Inactivated normal rabbit serum	Mouse carcinoma extract 0·4 c.c.	Guinea-pig kidney extract 0·4 c.c.	Goat erythrocyte extract 0·4 c.c.
1.	0·1 c.c.	m.	m.	c.
2.	0·05 ,,	n.c.	n.c.	,,
3.	0·025 ,,	c.	c.	,,
4.	0·01 ,,	,,	,,	,,

As the tables show, normal rabbit serum either does not contain complement fixing antibodies or does so to a very small extent.

The reaction carried out with sera immunised against mouse tumour, guinea-pig kidney and goat blood corpuscles however gave the following results:

TABLE III.

Complement fixation by goat erythrocyte antiserum with homologous antigen and guinea-pig kidney and mouse carcinoma extracts.

	Goat erythrocyte antiserum	Homologous extract 0·4 c.c.	Mouse carcinoma extract 0·4 c.c.	Guinea-pig kidney extract 0·4 c.c.
1.	0·1 c.c.	n.	n.	n.
2.	0·05 ,,	,,	,,	,,
3.	0·025 ,,	,,	sl.	,,
4.	0·01 ,,	sl.	m.	sl.
5.	0·005 ,,	m.	n.c.	m.
6.	0·0025 ,,	c.	c.	c.
7.	0·001 ,,	,,	,,	,,

TABLE IV.

Complement fixation by mouse carcinoma antiserum with homologous antigen and guinea-pig kidney and goat erythrocyte extracts.

	Mouse carcinoma antiserum	Homologous extract 0·4 c.c.	Guinea pig kidney extract 0·4 c.c.	Goat erythrocyte extract 0·4 c.c.
1.	0·1 c.c.	n.	n.	n.
2.	0·05 ,,	,,	tr.	m.
3.	0·025 ,,	,,	m.	c.
4.	0·01 ,,	,,	c.	,,
5.	0·005 ,,	m.	,,	,,
6.	0·0025 ,,	c.	,,	,,
7.	0·001 ,,	,,	,,	,,

TABLE V.

Complement fixation by guinea-pig kidney antiserum with homologous antigen and mouse carcinoma and goat erythrocyte extracts.

	Guinea-pig kidney antiserum	Homologous extract 0·4 c.c.	Mouse carcinoma extract 0·4 c.c.	Goat erythrocyte extract 0·4 c.c.
1.	0·1 c.c.	n.	tr.	n.
2.	0·05 ,,	,,	sl.	,,
3.	0·025 ,,	,,	m.	,,
4.	0·01 ,,	,,	n.c.	s.l.
5.	0·005 ,,	tr.	c.	m.
6.	0·0025 ,,	m.	,,	c.
7.	0·001 ,,	c.	,,	,,

Complement fixation tests with these sera gave with all three extracts positive results, although the sera deviated complement most strongly with their homologous antigens. In the case of goat erythrocyte serum this last is least marked, possibly owing to the removal of a certain amount of the more specific complement fixing antibody during the absorption with red cells.

II. HAEMAGGLUTINATION.

The question as to the relationship between haemolysis and haemagglutination does not seem settled yet. Ehrlich and Morgenroth (1913) who deny that haemagglutination is a prelude to haemolysis, as Bordet (1913) and Baumgarten (1913) believe, assert that both antibodies can coexist independently in the same antiserum.

Forssman (1911) has described haemagglutination on goat erythrocytes with guinea-pig kidney antiserum, but he did not give an exact account of his observations. It remained to be seen, therefore, if guinea-pig kidney and mouse tumour antisera contain haemagglutinins, as well as haemolysins, for goat erythrocytes.

The methods employed were as follows:

The immune sera were inactivated by heating in the incubator at 56° C. for half an hour. The 5 % suspension of red corpuscles was prepared in the same way as for the preceding experiments. To each of the test tubes containing diminishing amounts of inactivated immune serum made up to 1 c.c. with normal saline, 0·5 c.c. of 5 % erythrocyte suspension and 0·5 c.c. of normal saline were added. After incubation the tubes were placed at room temperature and the following morning the results were read, the degrees of haemagglutination being distinguished as follows:

+++ = very distinct
++ = distinct
+ = positive
± = doubtful
− = negative

The normal rabbit sera used in the complement fixation tests served as control.

TABLE VI.

Haemagglutination of goat erythrocytes by normal rabbit sera.

	1 c.c. of serum diluted	5 per cent. goat blood corpuscles	Normal saline	Result
1.	1 : 5	0·5 c.c.	0·5 c.c.	−
2.	1 : 10	,,	,,	−
3.	1 : 20	,,	,,	−
4.	1 : 40	,,	,,	−

Normal rabbit serum is thus seen not to contain haemagglutinin for goat erythrocytes at all.

The following tables show the same reaction carried out with various goat red cells, guinea-pig kidney and mouse tumour antisera.

TABLE VII.

Haemagglutination of homologous erythrocytes by goat erythrocyte antisera.

	1 c.c. of serum diluted	5 % goat blood corpuscles	Normal saline	Dilution	Goat erythrocyte serum		
					No. 1 A.U.=0·001	No. 2 A.U.=0·001	No. 3 A.U.=0·0025
1.	1 : 5	0·5 c.c.	0·5 c.c.	1 : 10	+ + +	+ + +	+ + +
2.	1 : 10	,,	,,	1 : 20	+ + +	+ + +	+ + +
3.	1 : 20	,,	,,	1 : 40	+ + +	+ + +	+ + +
4.	1 : 40	,,	,,	1 : 80	+ + +	+ + +	+ +
5.	1 : 80	,,	,,	1 : 160	+ + +	±	+
6.	1 : 160	,,	,,	1 : 320	+ +	−	−
7.	1 : 320	,,	,,	1 : 640	+	−	−
8.	1 : 640	,,	,,	1 : 1280	−	−	−

TABLE VIII.

Haemagglutination of goat blood corpuscles by guinea-pig kidney antisera.

	1 c.c. of serum diluted	5 % goat blood corpuscles	Normal saline	Dilution	Guinea pig kidney serum No. 1 A.U. = 0·001	Guinea pig kidney serum No. 2. A.U. = 0·005	Guinea pig kidney serum No. 3 A.U. = 0·005
1.	1 : 5	0·5 c.c.	0·5 c.c.	1 : 10	–	–	–
2.	1 : 10	,,	,,	1 : 20	–	–	–
3.	1 : 20	,,	,,	1 : 40	–	–	–
4.	1 : 40	,,	,,	1 : 80	–	–	–
5.	1 : 80	,,	,,	1 : 160	–	–	–
6.	1 : 160	,,	,,	1 : 320	–	–	–
7.	1 : 320	,,	,,	1 : 640	–	–	–
8.	1 : 640	,,	,,	1 : 1280	–	–	–

TABLE IX.

Haemagglutination of goat blood corpuscles by mouse tumour antisera.

	1 c.c. of serum diluted	5 % goat blood corpuscles	Normal saline	Dilution	Mouse carcinoma serum A.U. = 0·005	Mouse sarcoma serum A.U. = 0·02
1.	1 : 5	0·5 c.c.	0·5 c.c.	1 : 10	–	–
2.	1 : 10	,,	,,	1 : 20	–	–
3.	1 : 20	,,	,,	1 : 40	–	–
4.	1 : 40	,,	,,	1 : 80	–	–
5.	1 : 80	,,	,,	1 : 160	–	–
6.	1 : 160	,,	,,	1 : 320	–	–
7.	1 : 320	,,	,,	1 : 640	–	–
8.	1 : 640	,,	,,	1 : 1280	–	–

The tables show that goat erythrocyte antiserum certainly contains a haemagglutinin for goat blood corpuscles but that guinea-pig kidney and mouse tumour antisera do not. In other words, the receptors which are able to produce haemagglutinin for goat blood cells are not distributed in guinea-pig kidney and mouse tumours. One must therefore conclude that as the antigens for haemagglutinins and haemolysins for the same blood cells do not always exist side by side, but can be found separated from one another, haemagglutination is not necessarily a prelude to haemolysis.

SUMMARY.

By using watery extracts of mouse tumours and guinea-pig kidney, and alcoholic goat erythrocyte extract, it has been shown that mouse tumour, guinea-pig kidney and goat erythrocyte antisera contain complement fixing antibodies for all three extracts, although the reaction is most evident when the homologous antigens are employed.

Goat erythrocyte antiserum agglutinates goat blood corpuscles strongly while guinea-pig kidney and mouse-tumour sera do not contain haemagglutinin at all. Haemagglutination is therefore not to be regarded as a prelude to haemolysis; the antibodies for both processes can exist independently of one another. It has thus been seen that mouse tumour, guinea-pig kidney and goat erythrocytes have in common the property of producing, when inoculated into the rabbit, besides haemolytic amboceptors, complement fixing antibodies, but receptors for goat haemagglutinins cannot be found in either guinea-pig kidney or mouse tumours.

For his advice and assistance in carrying out this work I desire to thank Dr Schütze.

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N° 1

THE RELATION BETWEEN THE HATCHING OF THE EGGS AND THE DEVELOPMENT OF THE LARVAE OF *STEGOMYIA FASCIATA* (*AËDES CALOPUS*), AND THE PRESENCE OF BACTERIA AND YEASTS.

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Of the Lister Institute of Preventive Medicine.

INTRODUCTION.

The clearing action of mosquito larvae in turbid water and their scarcity or absence in clean water, is an old-established popular belief in mosquito ridden districts of both the old and new world. If the presence of certain species of *Anopheles* larvae in clear running water, where they doubtless feed on algae, be excepted, it is probable that this general observation is in the main correct. The fact that numbers of mosquito larvae are frequently present in the small collections of clear water which occur in the cut ends of bamboos, the axils of leaves, or the smaller rock or root pools, is an apparent but not a real contradiction of the correctness of this popular belief, because in these instances one of two possibilities may have occurred. Either the large number of dormant eggs which hatched when rain first filled the receptacle was sufficient to check and control bacterial or yeast development from the start, or, as is more likely in the case of root or rock pools, the turbidity due to bacteria or yeasts had been rapidly cleared as the rapacity of the quickly growing larvae increased beyond the source of nutriment.

Mitchell (1907), referring to *Stegomyia fasciata*, says it is pre-eminently a bacteria-eating "wriggler," presumably on the strength of the known fact that the larvae develop rapidly in sewage-contaminated water.

Howard, Dyar, and Knab (1912) mention bacteria as forming food for mosquito larvae in general terms, stating that the spores of algae, particles of dust, bacteria, protozoa, minute aquatic animals of many different kinds are swallowed. They specifically mention that the larvae of *Aëdes* are largely bottom feeders and may be seen vigorously working over dead leaves and vegetable débris with their mouth organs, apparently removing the fungoid growth which covers them.

Boyce and Lewis (1910) showed that the presence of mosquito larvae added to clean water led to an increase in the number of bacteria. Unfortunately the authors have not put on record the methods they pursued in arriving at this result, consequently we are left in doubt as to whether certain very necessary precautions to avoid fallacies were taken or not. For instance it is not stated in what stage or from what source the larvae they added to the clean water were taken, while, unless these factors were carefully controlled, there would be (as pointed out by the authors of the Carnegie Monograph on the Mosquitoes of North and Central America 1912) grave danger of the introduction of organic matter voided from the larval gut. The fact that an increase of bacteria followed the introduction of the larvae is therefore no valid argument against the popular belief of the clearing action of the mosquito larvae, unless the larvae had been introduced immediately after hatching, or just after a moult; the hatching of the eggs or moulting of the larvae having taken place in comparatively large quantities of clean water.

Bacot (1916) carried out a number of carefully planned experiments in Freetown, Sierra Leone, using newly hatched larvae of *S. fasciata*, with a view to ascertaining the amount and nature of the food consumed by the larvae and whether bacterial development in the breeding pans was a factor in the problem. He found that there existed a very definite relation between the development of bacteria and the growth of the larvae. Subsequently, on his return to England, he discovered, as detailed in a footnote to his report (see Experiments I and II), that water highly charged with organic matter, and swarming with bacteria, exerted a powerful stimulus to hatching on eggs which had not responded to immersion in clean water. The suggestion in his report that the bacteria themselves formed food for the larvae was based on the clearing action they displayed in water, rendered turbid by its enormous bacterial content, in conjunction with the fact that the gut-contents of larvae, taken from this water, showed only a few bacteria per field in contradistinction to the crowded fields displayed when water in which the larvae swam was examined. It being deemed impossible that bacteria could be excluded when the larvae were sweeping small particles of matter into their mouths, the explanation of their scarcity must therefore depend upon their rapid digestion.

A few rough experiments in which masses of bacteria and yeasts scraped from the surface of agar cultures were dropped into water containing larvae, showed that the larvae congregated round and greedily devoured them. Their jaws were evidently used to tear and disrupt the clumps of organisms, while the turbid cloud which formed round the struggling crowd of larvae was rapidly dissipated, presumably by the sweeping action of their mouth brushes.

Before describing the experiments and discussing their results, some preliminary explanations are necessary. For the sake of those readers who have no practical knowledge of mosquito breeding it is necessary to state that the conditions favourable for rearing the adults, though simple, require some nicety of adjustment for success, when the breeding pans are small. The essential points are temperature, the amount of nutriment in relation to the quantity of water, and the number of larvae in relation to both the latter factors. The temperature throughout the experiments under sterile conditions was 75° F. which if not quite high enough to give the speediest passage from egg to adult, is quite favourable for the species.

The amount of nutriment and number of larvae in proportion to the quantity of water were, however, in some of our experiments highly artificial.

In Bacot's report (1916) referred to above, the adjustment of these factors is dealt with from the aspect of the rearing of healthy adults. With the present research such careful adjustment was often neither possible nor necessary, the actual number of adults reared being altogether of secondary importance to the hatching of dormant eggs, and the relative speed of larval growth. Healthy conditions for the larvae occur when they are able to hold bacterial multiplication in check, when they do this very thoroughly their progress is slow and steady, if they hold it slightly in check their development is more rapid, while in cases where the balance of forces is delicate, larval growth is apt to be very rapid, resulting in a life or death race between them and the growth of bacteria. It mattered but very little if this balance, which could not in many cases be readily foreseen, resulted in the destruction of the larvae before the completion of their development owing to the exuberance of bacterial growth. While, although the lagging or eventual starvation of the larvae from lack of sufficient nourishment in some of the more narrowly adjusted experiments is regrettable, it need not lead to any confusion so long as it is borne in mind that the comparison of speed in growth must be made between the larvae in tubes of the same series and not with those in some other experiments in which the conditions or media differed.

With regard to the hatching of eggs, much preliminary work was done by Bacot in Sierra Leone which is fully detailed in his report (1916), but for the benefit of those who have not had any opportunity of perusing it, the main facts may be briefly recapitulated as follows:

The eggs of *Stegomyia fasciata* are small black spindle-shaped objects of 0·630 to 0·650 mm. in length, by about 0·160 mm. in width if of average size. The shell, which is highly chitinized, is covered with a delicate cellular reticulation and from the central area of a large number, if not of all, the cell spaces rise small rounded bosses, which sections of the egg show to be gelatinous, and not merely the bulgings of the chitin wall of the egg. The eggs are deposited by the females singly, either on the water surface or the wet margins of objects in or surrounding the water surface; no doubt capillary action is responsible for the stranding of many eggs. Incubation is complete after a period of 30 to 50 hours according to the temperature, and Bacot states that it is necessary that the surface of the egg be kept moist while incubation is in progress. McGregor (1916) seems also to have experienced this necessity. After incubation the eggs now containing living larvae may be dried and remain dry for many months, without losing their vitality. Hatching on immersion, after incubation and drying, is generally erratic, a few eggs or a number may hatch within a few minutes; some will take hours, while others will remain dormant under water for days, weeks, and even months, and yet eventually yield healthy larvae.

The *quantity* of water in which the eggs are immersed makes no appreciable difference, nor does the age of the female, or whether the eggs were of a late or early batch. Changing the water in which the eggs were laid had no effect when the fresh water was from the same source and of the same temperature, but a fall in the temperature of the water in which the eggs were lying, exposure to cool air for a few minutes followed by reimmersion in the same water acted as a stimulus to the hatching of a varied percentage of the dormant eggs. The effect of cooling, however, seldom caused more than a small percentage of the dormant eggs to hatch and sometimes had no effect. The immersion of incubated eggs before they had been allowed to dry and others of the same batch after drying, gave divergent results[1].

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[1] Although we have not yet followed this point up by planned experiments we have some reasons for supposing that the divergence may be due to bacterial action, during or immediately following incubation.

PRELIMINARY NOTES.

The experiments on which this paper is based were carried out in intervals of time during the course of other work, which in view of the war was of more immediate importance, although the actual delay which resulted from this cause was not serious, because, owing to the slow growth of larvae in some experiments, and the lengthy periods during which eggs remained dormant in others, slow progress was inevitable. From another aspect, however, the length of the gaps, extending in some instances over several months, between an initiatory experiment and its repetition, exerted a considerable influence on the final results, introducing an element of uncertainty as to the nature of the cause to which divergent results were due. For instance, the action of killed cultures and sterile extracts on dormant eggs is not consistent (in an earlier and later series of experiments) and it remains a matter of opinion whether the contradictory nature of the results arises from the treatment of the eggs, the conditions to which the parents were exposed, or the fluctuations in their heredity, which is possibly adjusted to the needs of climatic conditions in the natural habitat of the race of *S. fasciata* experimented with.

The bacterial side of the research was greatly hampered in that one of us had to drop all practical help in research owing to war work at a distance from the Institute, and, although he continued to act in an advisory capacity, it was not possible in his absence to follow various interesting points or to determine the species of the bacteria and yeasts which appeared in the course of the experiments.

It is perhaps necessary to add a word of explanation in regard to the use of the word "sterility." When working with a definite species or group of bacteria for which the suitable conditions of temperature and description of media are charted, the word "sterility" has a definiteness which hardly applies to this work. As here used the word refers to a failure of the organisms to make their presence apparent under circumstances conditioned to the needs of the mosquito larvae. Cultural tests for sterility were carried out with tubes of peptone broth and agar slopes for bacteria; in the case of yeasts with tubes of wort agar. In many cases experimental tubes which repeatedly gave sterile results when the inoculation was performed with a platinum loop were proved to be infected when the test was carried out with a Pasteur pipette which transferred 0·25 to 0·5 c.c., showing how slight the infection really was, either owing to the unsuitability of the temperature or medium for the organism, or more probably to the clearing action of the larvae. In other cases when infection was suspected owing to the progress of larvae in comparison with their fellows which had hatched from eggs of the same batch in other tubes, it was only proved when cultivations on agar were made with quantities of 0·25 to 0·5 c.c. and the tubes were incubated at 60° to 75° F. It is further significant that larvae from the same batch of eggs might in one tube make no progress at all while in a similar tube perceptible progress would take place, only to cease before the third instar was attained, raising a suspicion of the presence of an infecting organism which either died out or was killed off by the larvae, which subsequently starved. These facts suggested that when eggs hatch the conditions are seldom if ever *really sterile*, but that the organism responsible for the stimulus is unable to colonize the tube owing to small numbers or want of adjustment between itself and the conditions. On the basis of this suggestion the difference between hatching and no growth on the part of the larvae and slight growth preceding death would be explained by the yeast or bacterial infection, probably due to infection from the eggs, dying out immediately in the former instance and more gradually in the latter, possibly owing to the action of the larvae.

Nevertheless, in spite of our frequently correct suspicion, that larval growth indicated infection, there were a few cases in which adult insects were reared under sterile conditions—the final tests being to transfer the dead mosquito from the tube in which it had been reared and died to a tube of peptone broth in which it was allowed to remain for a week or more under a variety of conditions of temperature. In these cases only one or two adults resulted from several larvae, and the possibility remains that the real source of nutriment was obtained from their dead comrades. This last surmise is, however, at issue with the fact that the presence of dead larvae did not in most cases enable the living survivors to complete their development.

We desire to take this opportunity of recording our thanks and indebtedness to our colleagues on the Staff of the Lister Institute for their valuable suggestions and advice, especially to Dr Harriette Chick and Professor Harden, without whose assistance many of the experiments could not have been carried out.

METHODS.

Sterilization. One section of the experiments with eggs and larvae was carried out with unsterilized eggs, and larvae newly hatched from eggs put into clean water which were invariably found to be infected. In the other section attempts were always made to render the eggs sterile before use, and in spite of a number of probably unavoidable failures experience showed that with careful precautions it is quite possible to work under sterile conditions.

The following methods for sterilizing eggs were employed:

(*a*) Eggs were placed in a minute cotton bag, dipped into 2 % lysol for 5–10 minutes and washed in boiled water. The bottom of the bag was then snipped off and allowed to fall into a tube of broth.

This method gave a few successful results, in many cases, however, sterility broke down.

(*b*) Eggs were washed in warm (about 90° F.) soft soap and water for 15 minutes, then in 2 % lysol for 5–10 minutes; in boiled water for 5 minutes and then transferred by Pasteur pipette to tubes containing the sterile water or media.

This method gave many successful results, but there were some breakdowns in sterility, chiefly due to moulds and sporing bacteria.

(*c*) Eggs were washed in tap water by using a fine jet in a deep pan of water; they were then pipetted into weak lysol, about 0·5 %, and vigorously washed round by a jet; after a few minutes they were transferred to 2 % lysol and the stirring by jet repeated. After this they were again placed in 0·5 % lysol and then into boiled water.

Methods (*b*) and (*c*) were varied in minor detail from time to time.

(*d*) A method of sterilization by formalin vapour was tried, but it was too drastically applied in the first instance, the larvae dying on emergence from the egg. Although after suitable development it would possibly have given favourable results, it was not proceeded with, owing to the successful results obtained with (*c*).

Towards the close of the experiments it was found that the work of sterilization was rendered more certain, and less elaboration of the sterilizing process was necessary if more care was bestowed on obtaining eggs less liable to be covered with a number of species of bacteria, more especially those forming spores. Arrangements were therefore made to rear the parents from which it was proposed to obtain eggs for experiment under conditions which rendered the presence of an infection of sporing bacteria and moulds less likely. A breeding pan was carefully cleaned and a number of larvae were washed in five changes of tap water, by thorough agitation and violent currents set up by a water-jet; there is little danger of the larvae being washed out of a deep pan, as they invariably keep at the bottom when the water is agitated. They were then left 24 hours, so that they might void the contents of their guts, and then again washed in two or three changes of water. After this preliminary treatment they were put into the clean pan and fed exclusively on broth cultures of *Bacillus coli*. As a result of this procedure the percentage of breakdowns in the sterility of the experimental tubes was much reduced.

One or two precautions with regard to pipetting the eggs are also advisable. Floating eggs should be avoided, and single detached eggs alone should be taken up. When two or more eggs remain attached after immersion in the lysol there is a probability that at the point of attachment a portion of the surfaces will not have been completely sterilized. The reason for avoiding floating eggs is also the danger of incomplete sterilization. In order to get rid of eggs attached to each other, the eggs may be sifted through motor veil gauze or any other very fine gauze.

The whole of the experiments are placed as an appendix, references to them only being given in the text.

SECTION A. EGGS.

Effect of contaminated water. Unsterile conditions.

Experiments I and II, already referred to on page 483, show clearly the powerful action of water charged with·organic matter and living organisms on dormant eggs which failed to hatch in clean water.

Effect of alkaline solutions—under unsterile and sterile conditions.

Experiments III and IV. It was suggested by one of our colleagues that the results which followed the addition of water or other media charged with bacteria or yeasts might be due to the ammonia content. Aggramonte showed (1902) that the presence of lye from wood ashes stimulated the early hatching of the eggs. Two trials were carried out, one under unsterile and the other under sterile conditions. From these it seems quite clear that, although a few eggs may respond to the presence of alkaline solutions in either tap or distilled water, the effect is very feeble in comparison with that due to either contaminated water or a broth culture of *B. coli*.

Effect of acidity—sterile conditions.

Experiment IX. Early in the course of the experiments the possibility of the effects resulting from the addition of living bacteria to media such as peptone water or peptone broth being due to acidity was considered. A dilute solution of HCl adjusted to give an acid reaction similar to that in a tube in which the growth of bacteria caused the eggs to hatch produced no response during three days while the eggs were lying dormant in tubes of peptone water and peptone broth, but within 18 hours of the inoculation of these tubes with bacteria all the eggs hatched. A further trial with another tube of peptone water, belonging to the same series, in which the eggs had been dormant for 8 days, also showed a negative result during a period of 48 hours. The acid addition to this tube was increased so as to cause a much more marked reaction than that in the first trial. After inoculation with bacteria one egg hatched within 18 hours and another on the following day, and the remaining two on subsequent days. This halting response was apparently due to the slow and feeble development of the bacteria, owing to the extreme acidity of the tube. The larvae in the tube made relatively very slow progress.

We conclude from the results of the five experiments that the marked response of the eggs was due to the presence of bacteria or their products, apart from either the acidity or alkalinity produced.

Effect of introduction of different living bacteria into tubes of sterile media such as peptone water in which the eggs are lying dormant.

Staphylococcus pyogenes aureus. *Experiment IX* (page 506). A tube of peptone water in which eggs had been lying dormant for 15 days was inoculated from a culture of *S. aureus.* The bacteria failed to grow and the eggs did not hatch. Two days later the inoculation was repeated from the same culture, this time with success and the eggs all hatched within 16 hours.

Bacillus coli communis. *Experiment XI* (page 508). A tube of peptone water in which eggs had remained dormant for 11 days was inoculated from a culture of *B. coli.* All the eggs hatched within 18 hours.

Experiment XX. In a tube of sterile broth, made from dead insects, only one egg out of a number hatched. On the 34th day a quantity equal to about 1 in 7 of filtrate from a broth culture of *B. coli* was added. This had no effect on the dormant eggs, and the tube on the 39th day was inoculated from a living culture of *B. coli.* The dormant eggs hatched within a few hours. A similar result followed the inoculation of a tube of peptone broth in the same experiment with *B. coli*, the eggs hatching within a few hours of the introduction of the bacteria after they had lain dormant for 39 days.

In Experiment XXIII several instances occur. In a sterile tube of distilled water containing 1 part in 6 of a filtrate from a broth culture of *B. coli*, eggs that had been dormant for 28 days hatched within 12 hours after the tube had been inoculated from a living culture of *B. coli.*

In a sterile tube of distilled water containing 1 portion in 6 of beef broth the eggs hatched on the 50th day within a few hours after the inoculation of the tube with *B. coli.* A similar tube was infected on the 60th day with a small quantity of a broth culture of *B. coli*; the eggs hatched within 10 to 15 minutes. On the 100th day of the experiment eggs that had been lying dormant in a tube of sterile distilled water hatched within 10 minutes of the addition of 3 % of a living culture of *B. coli.*

In a duplicate tube the addition of 3 % of a recently killed culture of *B. coli* caused 1 egg to hatch out of 11 within 15 to 20 minutes, but no more hatched within an hour and a half, when the addition of 3 % of a living broth culture of *B. coli* caused 6 eggs out of the remaining 10 to hatch within 15 minutes. The remaining eggs did not hatch at all and were most probably dead.

Unidentified bacteria.

Cases in which hatching followed the breakdown of sterility were frequent; instances will be found in most of the experiments, in many cases two or more tubes containing different media being affected. Although the species of bacteria were not identified it was clear from their behaviour when sub-cultured that they were of different kinds; some developed spores, while at least three different species were present, which would only grow at a temperature below 80° F.

Apparently any species of living bacterium may act as a stimulus though there is some evidence that certain species act more quickly than others (see Experiment XXIV). It is also significant that hatching only follows inoculation with a small quantity on a loop after several hours, whereas if as large a quantity as 2 % or 3 % of a broth culture is added, hatching follows as a rule within 15 minutes. There is also some evidence which suggests that fresh cultures are more effective than older ones of two or three weeks' age.

Effect of the introduction of living yeast cells.

Experiment XI. To a tube of peptone water in which eggs had been lying dormant for 15 days, about 1 in 5 of a sterile watery extract of brewers' yeast was added. No result followed within one week, when the tube was inoculated from a living yeast culture. All the eggs hatched within a few hours.

Experiment XXII. Some of the eggs placed in a sterile tube of distilled water, with 1 in 6 of beef broth added, remained dormant for 28 days. A small mass of cells from a culture of *Saccharomyces cerevisiae* on wort agar was added, when the dormant eggs hatched within a few minutes.

In the same experiment 7 tubes of distilled water, in which the eggs had lain dormant for 28 days, were subjected to the same treatment, most of the eggs hatched within 5 to 10 minutes.

Experiment XXIII. Eggs that had been lying dormant for 28 days in a tube of sterile distilled water, containing 1 in 6 of the filtrate from a broth culture of *B. coli*, hatched within 10 minutes of the introduction of a small mass of living cells of brewers' yeast.

In the same experiment (XXIII) on the 100th day 3 % of sterile autolyzed yeast was added to a tube of sterile distilled water in which there were dormant eggs. One egg out of 9 hatched within 15 to 20 minutes; after an hour and a half, during which period no further eggs hatched, a small mass of living yeast cells from a wort agar culture was added, and the remaining 8 eggs hatched within 15 minutes.

These examples afford clear evidence of the stimulus to hatching exerted by living yeast cells. In Experiment XI the yeast used was one that had been isolated from a human throat, but in the other tests *S. cerevisiae* was used.

Effect of the presence of living moulds.

No special tests were carried out with moulds, but during the course of the experiments it was remarked that when a mould developed in an otherwise sterile tube many if not all the dormant eggs hatched. It is apparently possible for moulds to exert this influence when very small and quite inconspicuous. In a number of cases in which eggs had lain dormant for several days their hatching was quite inexplicable until a tiny white speck, which proved subsequently to be a growing mould, was noticed. A microscopic examination in one such instance showed the hyphae ramifying over the surface of the egg shells. Many examples of hatching in apparent response to moulds occurred in Experiment XXII.

The most plausible suggestion as to the nature of the stimulus which induces the eggs to hatch would seem to be that of *a scent* which penetrates to the larvae lying dormant within the egg shells causing them to make vigorous movements resulting in the uncapping of the egg. There is, however, a difficulty in the way of the acceptance of this theory, owing to the partial or entire failure in some cases of killed cultures or filtrates of *B. coli* and sterile yeast extracts to bring about the hatching of the eggs. This subject is dealt with in greater detail in the following section.

The effect of killed bacterial cultures; sterile filtrates of B. coli *and extracts of brewers' yeast.*

Until nearly the close of the experimental work no results had been obtained from the addition of killed cultures and sterile filtrates of *B. coli* (see Experiments XIV, XXI and XII). On one occasion (Experiment IX) the addition of about 17 % to 20 % of a sterile autolyzed extract of brewers' yeast caused eggs which had been lying dormant in a tube of peptone water for a month to hatch within one hour, but the larvae died within a few minutes. In Experiment XI about 8 % of a sterile watery extract of brewers' yeast was added on the 15th day to a tube of peptone water containing dormant eggs, but no action having resulted within a week, the tube was inoculated from the culture of a species of yeast isolated from a human throat. The eggs hatched during the following night.

When sterilized eggs were pipetted into tubes of distilled water containing 2 % or 3 % of an autolyzed extract of brewers' yeast (Experiments XVI and XVII) hatching was not general, nor did hatching occur any more freely than in different media in the other tubes. In Experiment XVII larvae continued to hatch out from dormant eggs over long periods. In one case eggs that had remained dormant for over 90 days hatched without any interference with the tube. While in another instance eggs that had been resting for 126 days hatched on the addition of boiled distilled water to three tubes which were almost empty owing to evaporation.

Experiment XXI. None of the eggs in two tubes of an autolyzed extract of brewers' yeast solution (3 % in distilled water) hatched within 96 hours, but one larva emerged in each tube within 120 hours. In one tube no more eggs hatched, but in the other two or three larvae emerged and died before the 12th day; both tubes were then inoculated from a living culture of *S. cerevisiae* and all the remaining eggs hatched during the night.

In spite of the foregoing negative evidence we thought it desirable to test the matter further, because it seemed possible that the failure might be due to either too high a temperature in the sterilization process or to the yeast extract not being sufficiently fresh—some days having been allowed to elapse between preparation and use.

With this object Experiment XXIV was planned. A number of tubes of distilled water containing eggs were used on the fifth day after the addition of the eggs. Cultures of *B. coli*, *S. aureus* and autolyzed extract of brewers' yeast which had been autoclaved (120° C.) were used, also cultures of *B. coli* and *S. aureus* which had been killed by steam heating at 100° C., a culture of *B. coli* killed by chloroform and a sterile filtrate of *B. coli*. The addition to the tubes was 6 %, except that in one of the tubes only 2½ % of the yeast extract was added, but this alteration made no difference to the result. It will be seen that in contradistinction to the earlier results the sterilized cultures and yeast extract were effective, but not the filtrate. This last was several weeks old at the time of use, but fresh filtrate had been used in the earlier trials without effect. It is interesting to note that the eggs were slower in responding to the *S. aureus* than to *B. coli* in all four tubes.

The results were so generally at variance with those in the earlier trials that it seemed probable that the difference must lie in the eggs. With a view to testing this hypothesis advantage was taken of several tubes of distilled water in Experiment XXIII in which eggs had been lying dormant for 100 days. These were tested by adding a quantity equal to about 3 % of a living culture of *B. coli*, autolyzed yeast sterilized by autoclave and *B. coli* sterilized by autoclave, the last two being from the same tubes as the additions to the tubes in Experiment XXIV. It will be seen that the results again differ, being more in line with the early trials, but not quite in conformity. There seems no doubt but that the susceptibility of the eggs varies greatly while it is also consistent with the results to suppose that the cultures but not the filtrate used—possibly because of their freshness—were more effective.

A further trial, Experiment XXV, was carried out to test the watery extract of yeast as against an autolyzed one, and both as against living cells from a culture of *Saccharomyces cerevisiae* on wort agar.

As a whole this test shows clearly that while, for these particular eggs, the autolyzed extract and the living cells were almost equally effective, the watery extract was far less so, and in one tube had no effect at all within three-quarters of an hour. The difference in the results in the several tubes must be taken as measure of the variable susceptibility of the eggs. It will be noted that in one of the tubes to which the *living yeast cells* were added, there was only a partial response within the first three-quarters of an hour. It is regrettable that no tubes containing eggs which had long lain dormant were available to control results, as was done with the previous trial (Experiment XXIV).

In discussing these results it is necessary to note that hatching may follow if during the sterilizing process the eggs are left too long in lysol. Sometimes these eggs do not uncap until after their transference to water, but in both cases the larvae fail to come right out of the shell. The same phenomena followed when eggs were exposed to formalin vapour for several hours. After transference to water large numbers uncapped, but the larvae only partially came out of the shells. In these instances no movement on the part of the larvae was ever observed. It is possible that they were killed within the egg, but cut or partially cut the cap in their dying struggles. There is a difficulty in the way of the acceptance of this explanation, however, because it is not easy to understand why, in the case of those placed in lysol, the uncapping should sometimes be delayed until after they were transferred to water.

The differential hatching of the eggs may, as pointed out in the introductory notes, arise as an hereditary trait owing to an adaptation to meet the needs of seasonal changes. On the other hand there is evidence that treatment during incubation or prior to full immersion may be the cause of more ready response to immersion. Bacot (1916) found when in Freetown that of a given batch of eggs, which had incubated on a wet surface, 98 % of those which were immersed without allowing them to dry hatched within 30 minutes; of a portion of the same batch placed in water after 24 hours' drying the hatching of 84 % was distributed over a period of 65 days; while the balance of the batch, which was kept dry for 7 days before immersion, again showed a divergent result, 43 % hatching within 24 hours, another 11 % following within 51 days and of the balance 8 % containing living larvae when dissected after 81 days.

In Experiment XVI the eggs were not allowed to dry before sterilization, as it was desired to test the theory that a check in the influence of bacterial action on the eggs might be the cause of delay in their hatching when subsequently immersed. Although the larvae did as a matter of fact emerge rather freely from these eggs under sterile conditions, the evidence from Experiment XXIII, in which also they were not allowed to dry before sterilization, is entirely negative, the number hatching under sterile conditions being small; while in Experiment XXV in which dried eggs were used the percentage hatching in some of the tubes was considerable. It is probable that the problem is a complex one, two or more factors being concerned.

The results of the experiments on eggs is so far clear and decisive in regard to the fact that the presence of bacteria, yeasts and, less definitely, moulds, does exert a stimulus causing eggs to hatch that would, apart from their presence, have remained dormant for a longer period. It also seems definite that this stimulus is less powerful, or may be altogether ineffective, if killed cultures or sterile filtration and extracts of bacteria or yeasts are used. The difference is apparently one of *quantity* not *quality*, its extent being chiefly dependent upon some variation in the susceptibility of the eggs.

SECTION B. LARVAE.

Unsterile conditions.

The fact that the larvae greedily consume clumps of bacteria or yeasts (*S. cerevisiae*) removed from agar cultures has already been mentioned in the introductory remarks. This rough test is open to the objection that what the larvae struggled for was not the organism but the organic matter on which they were living, or by-products produced in their development.

In Experiments V and VI, *B. coli* washed by centrifuging were given as food to a batch of larvae, while to an equal number in a similar quantity of water no food was supplied; the unfed batches constituting controls. The results show conclusively that the larvae cannot only live, but thrive on the bodies of bacteria.

Sterile conditions.

These early trials, followed by a series of experiments (VII, VIII, IX and XI), afford examples, in which the eggs were sterilized and transferred by Pasteur pipette into tubes containing various sterile media, the object being to ascertain if it was possible for the larvae to develop under sterile conditions. In the main the results are clear and decisive; while sterility is maintained, larval development is either inhibited or proceeds excessively slowly in conjunction with a high, or more frequently a total mortality. The infection of a tube with bacteria is generally followed by larval progress, usually rapid. The only exceptions are cases in which the larvae had existed under sterile conditions for long periods and seemed incapable of adjusting themselves to the change. Exceptions to the general rules of rapid larval development in infected tubes are most noticeable in Experiments XII and XIII when the bacteria which caused the breakdown in sterility proved to be feebly growing species, which altogether failed to keep pace with the larval requirements. In Experiments XI, XVI and XVII adults were reared under sterile conditions, the greatest success (in Experiments XI and XVI) being attained in a medium of pure beef broth (without the addition of salt or peptone) which for some unexplained reason seemed favourable for slow but sustained growth. Two adults were reared in separate tubes of a 3 % solution of an autolyzed extract of brewers' yeast in distilled water (Experiment XVII). One of these was reared under sterile conditions and the other ought, properly speaking, to be credited to these conditions as, although a mould was present in the tube when the adult emerged, all the evidence is against any suggestion that the larvae obtained any nutriment from this source —the presence of moulds being seemingly inimical or neutral to larval development; in this particular instance all the remaining larvae in the tube died.

While in these, and in fact in all, cases of larval progress under what are apparently sterile conditions, the possibility must not be excluded of a slight infection occurring during a portion of the larval existence and then dying out, the only evidence in support of such a supposition is the fact that larval progress varied in what were apparently sterile tubes independently of the media. This has happened in one or more out of a batch of tubes of the same medium, the eggs being part of a number all sterilized together. Taking into consideration the variability exhibited by the eggs at a stage when they contain fully developed larvae, it will require a considerable body of positive evidence based on carefully planned experiments before such a possibility can be accepted as a probability.

In Experiment XI a test was carried out to see if a change from one sterile medium to another had any effect in stimulating larval growth. Of two larvae withdrawn on the 15th day from a sterile tube of peptone broth, one was transferred to a tube of broth made from dead insects and the other to a tube of water in which horse dung had been steeped. After a pause of some 48 hours some evidence of quickened growth was observed, but the progress was not continuous and both larvae died some 50 days later without having completed their larval development.

Experiments XII and XIII were designed with a view to trying sterile media composed of substances more likely to be met with by mosquito larvae under natural conditions than those normally used for bacteriological purposes. Tubes containing water in which horse dung had been steeped 48 hours previously and a broth made from dead insects—chiefly flies—were used. The series afforded but little evidence bearing on the original purpose of the tests owing to the infection of the tubes by obscure species of bacteria whose presence was difficult to detect, and such evidence as was forthcoming was negative. The tests convinced us, however, of the unsuitability of these media which were likely, owing to the sources from which the nutriment contained in them was drawn, to harbour sporing bacteria.

Particulate matter. One of our colleagues suggested the possibility of the necessity of particulate matter in the medium as it seemed possible that larvae swimming in a true fluid might be unable to pass it through the alimentary system in the absence of solid particles in the intestine. We accordingly added powdered animal charcoal to a number of tubes of a solution of autolyzed yeast in distilled water (Experiments XVI and XVII) with apparent success at the outset, as the larvae not only ingested it—the dark gut content being clearly visible—but made quicker progress than others in the same media without the charcoal. The advantage did not, however, continue and, although one reached the pupal stage, no adults were reared; on the other hand, as already recorded, one adult was reared from a sterile tube of the yeast extract solution, without the charcoal. It is also to be noted that the insect broth which contained much solid matter also failed, under sterile conditions, to confer any advantage after the first few days.

A few trials were carried out to test if milk acidified to such a degree that the colloidal particles of the casein were increased in size but not precipitated and white of egg precipitated as very minute particles, would afford the necessary pabulum for the larvae. After sterilization small quantities of these preparations were added to media in which eggs had already hatched, but in which the larvae were making no progress, or the larvae were transferred by Pasteur pipette to tubes containing milk or white of egg in this form. Examples will be found in Experiments XIII, XVIII, XIX and XX. In no case did the addition or presence of the substances enable the larvae to develop while sterility was maintained; although in some instances slight progress followed the addition (Experiment XIX, page 518), it soon ceased, the larvae eventually dying without attaining their full growth.

Killed cultures of B. coli.

In Experiment X a preliminary trial was made to ascertain if the larvae could be reared under sterile conditions upon a culture of *B. coli* killed by heat. No success followed, but young larvae hatched from unsterilized eggs made rapid progress in a control tube, this tube being of course infected by the larvae. Experiment XIV was a repetition on a larger scale; several tubes were prepared, but the eggs placed in these failed to hatch. A single newly hatched larva (the only one which could be spared at the moment) was transferred from another experiment to one of the tubes. It failed to make any progress during a month and died within a few hours of the inoculation of the tube at the end of this period.

Sterile filtrate of B. coli.

In Experiment XXIV a culture of *B. coli* killed by chloroform was added to tubes of sterile distilled water containing eggs; a number of these hatched and tube No. 12 remained sterile, although its fellow No. 11 became infected on the 11th day. None of the larvae in No. 12 passed the first moult, while some of those in the infected tube had made considerable progress and were in the third instar. The larvae in tube No. 12 all died in the second skin, whereas in the infected tube the larvae were well grown before the food supply failed, two at least having attained their fourth (last larval) instar.

Thinking that possibly the action of the heat used in killing the cultures of *B. coli* might have the effect of destroying its nutrition value for the mosquito larvae, a number of trials were carried out with the sterile filtrate from broth cultures of *B. coli*. No success attended any of them, either in neat or diluted condition, nor did the addition of the filtrate to other sterile media in which larvae were living produce any effect, apart from the few cases in which sterility broke down at the period when the filtrate was added. (See Experiments XIX, XX and XXIII.)

Living yeast and yeast extracts.

See Experiments XVI, XVII and XXI; note also the conclusion of XXII, page 521, of XXIII and of XXIV and the conclusion of XXV. There is a break of similar character between the nutritive value of living *S. cerevisiae* and sterile extracts of brewers' yeast to that which separates living from dead cultures, or sterile filtrates of bacteria. It is not quite so definite, however, as the larvae do make progress in solutions of autolyzed yeast extract, whereas throughout the whole course of the experiments no larval progress has occurred in sterilized cultures of bacteria or sterile filtrates. The reason for this difference is not obvious and it seems possible that by some system of concentrating and extracting bacterial cultures analogous to the autolyzing process with yeasts, the difference might be eliminated.

The effect of the presence of moulds on larval growth.

Although no experiments were planned with a view to testing the influence of moulds, their occurrence in both sterile and bacterially infected tubes was so frequent in some of the experiments that a considerable amount of general observation was possible. The impression gained during the progress of the research was that moulds were inimical to larval development, but a careful consideration and comparison of the notes show that the fatality which so frequently followed their appearance in the experimental tubes cannot be clearly demonstrated as due to their presence. In a few cases of rapidly spreading surface moulds it is extremely probable that they were an accessory, if not the actual cause of death, and in Experiment XXII an instance of mortality apparently due to moulds occurred in tubes Nos. 4 and 5 of the distilled water series. What does, however, appear quite definite is that they are of no service whatever to the larvae as a source of food supply. In fact it seems reasonable to suppose that they may compete with the larvae for whatever supply of nutriment is available. Although the evidence on this point is not altogether consistent, in Experiment XXII it appears quite negative, there were several instances in which fully grown larvae died in tubes where moulds had developed. In a few cases one or two out of several of the larvae succeeded in completing their development, the others dying rather inexplicably if the moulds were not implicated. Two or three instances occurred of moulds growing from the mouths of dead larvae, as though swallowed spores had developed in the alimentary canal; while there is of course no evidence that the development of the mould preceded death, the spores must presumably have been ingested by the living larvae. Notes referring to the presence of moulds will be found in Experiments XXI, XXII and towards the close of XVII and XXIII.

Our conclusion respecting the evidence as a whole may be stated as follows: that the larvae of *Stegomyia fasciata* greedily consume both bacteria and yeasts on which they can thrive in the absence of any other food, whereas in very many instances they fail entirely to develop on a variety of nutritive fluids and particles, including dead bacteria, under sterile conditions. In the far less numerous cases where progress was made under apparently sterile conditions, growth was always relatively very slow to that under otherwise equivalent but unsterile conditions, and the mortality was very high. The rearing of adults under sterile conditions being so exceptional we feel justified in stating that the presence of bacteria or yeasts is a practical necessity for the maintaining of the species. If one considers the facts shown by Bacot (1916) regarding the scarcity of bacteria in the alimentary canal of larvae taken from water swarming with bacteria in relation to the exclusively browsing habit of the young larvae and its partial retention by the older ones when feeding, together with the experimental results above recorded, there seems good reason for supposing that bacteria and yeasts afford the chief food supply of the larvae. The ingestion of larger particles and the structure of the jaws are not incompatible with this view, because such particles are likely to be covered with a bacterial growth while the jaws are of use in gnawing away portions of decaying organic matter. They were evidently used to disrupt clots of both bacteria and yeasts in the preliminary experiments mentioned in the introduction.

It seems probable that this knowledge may be of assistance in the destruction of this species of mosquito, as it should enable the methods now so largely available for the purification of water from bacteria to be utilized. On a minor issue it may be of service in enabling Sanitary Officers to escape the annoyance caused by the failure to breed more than one or two isolated species from jars containing mosquito larvae captured by their inspectors. This failure no doubt arises owing to starvation, because the bacteria are killed by the action of light, it being customary to stand jars with wrigglers in the full light of a window. There is the further possibility that the eggs after a careful and probably lengthy research might be found to be of service as a rough and ready means of testing the relative purity of water in regard to living organisms.

EXPERIMENTS WITH UNSTERILIZED EGGS.

Effect of foul water charged with bacteria.

Experiment I.

100 c.c. of tap water was put into each of two glass pans. The water temperature during the course of this experiment was about 65° to 67° F.

A batch of about 350 eggs laid on filter paper three weeks previously and allowed to dry after incubation, but stored in a moderately humid atmosphere, was divided into two lots, approximately half being immersed in each pan.

To the water in one pan (*A*) was added 3 c.c. of sewage water (a fragment of human faeces was placed in water and allowed to incubate for 48 hours at 80° F.); to the water in the other pan (*B*) nothing was added.

After 1 hour in *A* 4 eggs had hatched.
,, ,, *B* no eggs had hatched.
After 4 hours in *A* 10 additional eggs had hatched.
,, ,, *B* no eggs had hatched.
After 20 hours in *A* 113 additional eggs had hatched.
,, ,, *B* 6 eggs had hatched.
After 70 hours in *A* 9 additional eggs had hatched. Total 136.
,, ,, *B* 36 ,, ,, ,, Total 42.

Experiment II.

The experiment was repeated, using water in which horse dung had been steeped for the stimulating fluid. The quantities and other arrangements were similar to those in Experiment I, 3 c.c. of the contaminated water was added to pan *A* at the start, but none to pan *B*.

After 18 hours in *A* 148 eggs had hatched.
,, ,, *B* 11 ,, ,,

3 c.c. of the contaminated water was then added to pan *B*. At the expiration of another 18 hours 159 additional eggs had hatched in pan *B*. The size of these larvae suggested that they must have hatched within an hour or two of the addition of the manure water.

Experiment III.

Contrasted effect of alkali and water charged with bacteria.
Temperature 75° F.

Into each of 4 pans containing 100 c.c. of tap water was placed ·007 gr. of incubated eggs of *S. fasciata* (the number would be about 200 or 300 eggs). The pans were kept all night at 75° F. Pan (1) formed a control. To pan (2) was added a solution of washing soda, producing an equivalent of 1 in 1000 in the pan; to pan (3) sufficient ammonia was added to produce 1 in 1000. To pan (4) about 5 c.c. of peptone broth from an experimental tube in which a bacterial infection caused the eggs to hatch.

After 6 hours (No. 2) Soda, showed a few larvae out.
,, (No. 3) Ammonia, showed a few larvae out
,, (No. 4) Contaminated, many.
,, (No. 1) Control, only one or two.

To the control pan about 2 c.c. of an infusion from horse dung was added, and the pans were left all night.

18 hours later (No. 2) Soda, showed a small number out.
,, (No. 3) Ammonia, about the same number out.
,, (No. 4) Contaminated, swarms.
,, (No. 1) Control, after the addition of the infusion of horse manure many more had hatched than in either the soda or the ammonia containers, but not so many as in the contaminated pan.

10 c.c. of water from pan (No. 4) was added to pans Nos. 2 and 3. An examination 6 hours later showed that the numbers of larvae had increased by one-third to a half, the freshly emerged larvae being easily distinguished from those which had hatched some hours previously, by their pointed conical heads.

EXPERIMENT IV. *With sterilized eggs.*

Contrasted effect of alkali, filtrate of B. coli *and living* B. coli.

Tubes taken from Experiment XXI (see page 519).

A batch of eggs was sterilized by Method *b* and from 12 to 20 were pipetted into a number of tubes containing 10 c.c. of sterile distilled water and one (No. 5) containing sterile filtrate of *B. coli*. After 20 hours at 75° F. the tubes were examined and four containing distilled water were selected in which only one or two eggs had hatched, the idea being to use resistant eggs which did not respond without stimuli.

In tube No. 1, 2 eggs had hatched (1 larva was dead).
,, No. 2, 2 ,, ,,
,, No. 3, 2 ,, ,,
,, No. 4, 1 ,, ,,
,, No. 5, 0 ,, ,,

To No. 1 was added 1 c.c. of water in which horse dung had been steeped for 48 hours at 37° C. ... ... = 10 %.
,, No. 2 was added 1 c.c. of a sterile filtrate of a *B. coli* culture = 10 %.
,, No. 3 ,, 1 c.c. of a 1 % solution of washing soda = 1–1000.
,, No. 4 ,, 1 c.c. of a 1 % solution of ·880 ammonia = 1–1000.
No. 5, a tube of filtrates of *B. coli* culture, was inoculated from a living culture of *B. coli*.

Result after 1 hour:

	Increase in hatching
No. 1, more than 12 larvae out	10 or over
No. 2, 4 larvae out	2
No. 3, 3 ,, ...	1
No. 4, 1 ,, ...	0
No. 5, 0 ,, ...	0

After 24 hours:

	Increase in hatching
No. 1, more eggs had hatched—probably all.	
[1]No. 2, 8 larvae out	6
No. 3, 3 ,, ...	1
No. 4, 2 ,, ...	1
No. 5, 12 ,, ...	12

After 68 hours:

No. 1, all the eggs hatched, larvae have grown rapidly.
No. 2, 9 larvae out—slight progress.
No. 3, 4 ,, no progress.
No. 4, 2 ,, both dead.
No. 5, all (12) larvae out, have grown rapidly.

The strength of the soda and ammonia solution in tubes Nos. 3 and 4 was doubled to 1 in 500.

After 24 hours no further eggs had hatched.

To each tube, Nos. 2, 3 and 4, ·5 c.c. of a culture of *B. coli* was added.

One hour later: all the remaining eggs in tubes 2, 3 and 4 had hatched—

No. 2, 12 or 13 living larvae.
No. 3, 12 or 14 living larvae.
No. 4, 12 living larvae.

The larvae in No. 4 (ammonia 1 in 500) all died within 24 hours.

Two days later the larvae in tubes Nos. 2 and 3 had made very rapid progress and had caught up to those in tube No. 5 in the matter of growth. They actually passed them during the next day or so and then slowed down. The larvae in all these tubes came to a standstill within 10 days, having apparently exhausted their food supply.

EXPERIMENT V (*unsterilized conditions*).

Ability of the larvae to develop on a diet of bacteria.
Temperature 75° F.

Bacillus coli was washed by centrifuging 4 times in succession and a small quantity of the precipitate placed in 30 c.c. of tap water with 5 newly-hatched larvae.

Control. 5 newly-hatched larvae of the same batch were placed in 30 c.c. of tap water, minus the *B. coli*.

The control larvae marked time with little or no progress; 1 larva died on the 6th day and 1 was feeble and died on the 8th day. By the 11th day 2 of the larvae passed the first moult, while a third larva died on the 12th day. After 17 days 1 larva attained to its third instar; both the larvae were living after the 30th day, but were dead by the 37th day. The larvae given *B. coli* grew rapidly; by the 3rd day 2 of the larvae had passed the second moult and the others were nearly full grown in their second instar.

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[1] It is probable that tube No. 2 was already infected as numerous later tests of *B. coli* filtrate added to tubes in which dormant eggs were lying, showed that the filtrate did not stimulate hatching.

The supply of washed *B. coli* in the pan was renewed. By the 6th day all the larvae were in the fourth instar. One pupated on the 8th day, and the remainder had pupated by the 10th day. Two ♀♀ specimens emerged on the 12th day and two more ♀♀ and a ♂ by the 13th.

The pan was then restocked with 5 newly-hatched larvae, but no fresh food given. All the larvae were in their second instar by the 3rd day; they reached the third instar by the 7th day, having exhausted the food supply, and were still in the same stage 10 days later.

EXPERIMENT VI (*unsterilized conditions*).

Ability of larvae to develop on a diet of bacteria.
Temperature 75° F.

Into one of two beakers, each containing 200 c.c. of tap water, was placed sufficient of a washed culture of *B. coli* to cloud the water.

20 newly-hatched larvae were placed in each beaker, and card covers placed on top to exclude dust.

Control. No appreciable progress, beyond the expansion of the head, took place within a week, then 8 died within 24 hours. Seven days later only 6 of the 20 larvae were living, 3 of which had passed the first moult. After another week (21 days from start) these 6 were still living, and making slow progress.

After 75 days there were two survivors, which by this time were slender larvae in the third instar. A little water mixed with faeces and therefore containing bacteria was added to the pan. Rapid growth was noticeable by next day; 1 larva had pupated within 5 days, and by the 9th day—84 days after hatching—2 adults were bred.

The larvae given *B. coli* made rapid progress; all were in the third instar by the 4th day; 16 had pupated by the 9th day; 4 ♂♂ and 1 ♀ emerged on the 11th day and the remainder followed within a few days; the last, which had lagged in the larval stage, taking 16 days.

Experiments under sterile conditions in tubes containing nutrient media.

EXPERIMENT VII.

Eggs sterilized by Method *a* were pipetted into a tube of peptone broth, after a night at 75° F., the tube was allowed to cool to 60° F., and some of the eggs hatched. The larvae showed no sign of growth after 48 hours at 75° F. The tube was then infected with *B. coli*; within 18 hours at 75° F. the broth was cloudy and the increase in size of the larvae was very obvious.

A curious feature of the experiment was that small bubbles of gas which were liberated by the fermentative action of the bacteria, formed on empty eggs, fragments of egg-shell etc. lying at the bottom of the tube, floating them up to the surface, when, the bubble becoming detached, they sank to the bottom and the process was repeated. Similar bubbles formed at the apex of the syphon tubes of the larvae, causing them to float upwards in spite of their struggles to swim downwards. So regular and constant was this process that the larvae got but little time for undisturbed feeding, and their growth was slow in comparison with their rapid start.

After 4 days the formation of gas ceased and the larvae again commenced to grow rapidly, and within 3 days some had attained their third skin. The tube, however, became very foul and all the larvae were killed.

EXPERIMENT VIII.

Eggs sterilized by Method *a* were pipetted into a tube of peptone water and a tube of beef broth without either salt or peptone.

No eggs hatched in either tube in response to the conditions and lapse of time, which were successful in the first trial.

Broth. After 48 hours sterility broke down in the broth tube; many of the eggs hatched and the larvae made rapid progress, 4 days later some of the larvae passed their third moult, but others were much behind on the 24th day of the experiment; 22 days after hatching the first adult was reared and others followed, but there was a heavy death-rate, presumably owing to there being too gross a growth of bacteria in the tube.

Peptone water. This tube remained sterile and, in spite of warming and cooling, no eggs hatched until the 3rd day, when one larva emerged; others followed and 5 or 6 were seen by the 5th day. Growth was not quite inhibited, but hardly any progress had been made by the 4th day after hatching, the larvae being still in the first instar.

On the 8th day of the experiment, 4 days after the hatching of the eggs, sterility broke down, the tube becoming cloudy. The larvae emerged from the remaining unhatched eggs and rapid progress commenced.

The larvae made rapid growth at first, and, owing to their number or the less vigorous growth of the bacteria, the conditions in the tube remained healthy, the larvae keeping the clouding of the peptone water in check and finally clearing it; they then began to find a shortage of food and ceased to grow, finally dying on the 24th day of the experiment—the last to die having reached the third instar.

EXPERIMENT IX.

(Includes notes respecting the addition of acid on dormant eggs.)

Eggs were sterilized by Method *b* and pipetted into four tubes of peptone water and four tubes of broth, only 5 or 6 eggs being placed in each tube.

After 48 hours, 4 eggs had hatched in one of the tubes of broth (No. 1) and in this tube only had sterility broken down; a 5th larva emerged in this tube next day. A plate culture was made from this tube, and a sporing bacillus isolated. On the evening of the 3rd day from the start one of the broth (No. 2) and one of the peptone tubes (No. 3) in which no eggs had hatched, were inoculated from the tube which had become infected. By next morning 4 larvae had hatched in the broth and 1 in the peptone tube. None hatched within the next 2 or 3 days in the remaining sterile tubes. The following test was then made: to two of the sterile tubes—broth No. 4 and peptone No. 5—was added sufficient of a dilute solution of HCl to produce a similar acid reaction to the broth tube in which sterility broke down. No response took place within 20 hours, but the remaining eggs in the inoculated peptone water hatched within this period.

By the 7th day of the experiment the larvae in the broth tube in which sterility broke down were all in the fourth instar, while those in the inoculated broth tube were in the second and those in the inoculated peptone water in the third instar. No eggs had hatched in the tubes to which acid had been added 3 days previously and the tubes were now inoculated from No. 1; within 18 hours all the eggs in them hatched.

Another trial was made, with dilute acid of higher concentration, in one of the peptone tubes (No. 6) in which the eggs had now lain dormant for 8 days; the acid reaction after the addition being much stronger than in the original broth tube (No. 1).

After 48 hours, during which period no larvae hatched, the third tube to which acid was added (No. 6) was inoculated from tube No. 1; by next morning (18 hours later) one egg had hatched, another larva emerged on the 2nd day, and 2 others on following days. The bacterial growth in this tube was feeble, presumably owing to its relatively strong acidity, while the larvae in it made very little progress.

On the 14th day of the experiment two eggs were taken by means of a Pasteur pipette from the remaining sterile broth tube (No. 7) and transferred to a tube of peptone broth; they lay unhatched in the tube of peptone broth for 8 days, when the tube was inoculated with *Staphylococcus aureus*; within 16 hours larvae had emerged from both the eggs. They died, however, within 3 days, apparently owing to the vigour of the bacterial growth.

On the 15th day of the experiment, the broth tube from which these eggs had been removed (No. 7) was inoculated with *S. aureus*. The culture did not grow and the eggs failed to hatch. Two days later the inoculation was repeated from the same culture; this time with success, and the eggs which had been lying in the broth for 17 days hatched within 16 hours.

On the 32nd day of the experiment 1 c.c. of sterile autolyzed extract of brewers' yeast (= 17 % to 20 %) was added to the remaining tube of peptone water (No. 8) in which the eggs had been lying dormant for a month. The eggs hatched within an hour, but the larvae died within a few minutes of their emergence.

History of the larvae in the above experiment.

Tube No. 1; eggs hatched in response to casual infection.

The larvae were in the fourth instar by the 7th day; 4 days later the first pupa was noted and 3 days later the first pair of adults emerged; 2 ♂♂ and 3 ♀♀ followed within the next day or two—15 days from hatching.

Tube No. 2 broth and No. 3 peptone eggs hatched on the 3rd day of experiment. The bacterial growth was more vigorous in the peptone water than in the pure beef broth. Four days after the hatching of the eggs the larvae in tube No. 2 were in their second instars, while those in tube No. 3 were already in the third skin. This rapid progress on the part of the larvae in tube No. 3 (peptone water) did not continue long; the dense growth of bacteria rendered the conditions unhealthy and 4 days later the larvae in tube No. 2 were ahead of those in tube No. 3, while the latter were beginning to die off.

On the 11th day from the hatching of the eggs all the larvae in tube No. 3 were dead, while two of those in tube No. 2 had pupated. The remaining larvae were full fed and the broth was bright and transparent, the number of bacteria having been reduced to a minimum either as a result of the larvae devouring them or organic matter in the broth necessary for bacterial development.

Tubes Nos. 4 and 5. Four days after hatching, the larvae had made good progress, those in the broth tube being well ahead, while by the following day the growth of bacteria in the peptone water had become exceedingly dense and one or two of the larvae were dead. By the 7th day after hatching 4 larvae in the broth tube had pupated, while the surviving larvae in the peptone water were still in their third instar. Five days later (12 days from hatching) all the larvae in the peptone water were dead, and a pair of adults had emerged from the pupae in the broth tube; the remaining pupae and larvae died.

Tube No. 6. These larvae made very slow progress; the growth of bacteria in the tube being feeble. By the 6th day after hatching they had only attained to their second instar. Five days later the larvae were still all living and had made steady progress; one pupated on the 13th day from hatching and 2 ♂♂ emerged on the 17th day; 2 ♀♀ following on the 18th and 20th days.

Tube No. 7 (broth). Four days after hatching only 2 of the 4 larvae were living; these had made rapid progress and were in their fourth instar; 2 ♂ specimens were bred on the 11th day from hatching.

EXPERIMENT X.

Washed B. coli *killed by heat as food for the larvae.*

A small quantity of washed *B. coli* was put into two tubes of distilled water which were sterilized by heat.

Into one tube a larva was transferred from a tube of peptone water that had remained sterile for several days; into the other several larvae that had just emerged from unsterilized eggs placed in tap water. The cloudiness which followed in this tube indicated that the larvae had infected it. The growth of these larvae was rapid; on the 5th day they were well grown in the third instar—the sterile larvae having made barely appreciable progress in the first instar.

The progress of the infected larvae slowed down gradually as the water cleared, but they continued healthy.

In the sterile tube the larvae progressed very slowly and died after moulting on the 9th day.

EXPERIMENT XI.

(Includes notes concerning the effect of sterile yeast extracts on dormant eggs and a change of diet, under sterile conditions, on the larvae.)

Five to 7 eggs sterilized by Method *b* were transferred by pipette into the following tubes: 4 of peptone broth; 3 of peptone water; 3 of beef broth (no salt or peptone); 1 in boiled tap water and 1 in boiled distilled water.

After 24 hours eggs left in the lysol used in the sterilizing process had hatched; the larvae were dead and many had failed to get free of the eggshell. Only one egg hatched out of a number that were transferred from the lysol to tap water. Several eggs out of a large number left in the flask of boiled water hatched. Some eggs also hatched in the tubes of peptone broth, broth tubes, and in one of the peptone water tubes, but none in the boiled tap water or boiled distilled water.

After 48 hours one larva hatched out in the boiled distilled water; none in the boiled tap water.

During the same period in peptone water two larvae came out of 6 eggs in one tube only.

Beef broth. Tube (No. 1), 1 out of 7 eggs
,, (No. 2), 3 ,, 7 ,,
,, (No. 3), 4 ,, 7 ,,

Peptone broth. Tube (No. 1), 5 ,, 6 eggs (1 larva dead)
,, (No. 2), 4 ,, 6 ,,
,, (No. 3), 2 ,, 5 ,,
,, (No. 4), 2 ,, 6 ,,

The growth of the larvae was negligible.

Three days later (5 days from start):

Boiled tap water. None had hatched.

Boiled distilled water. No progress by the larva.

Peptone water. No further hatching; no progress by the larvae already hatched.

Beef broth. Tube (No. 1), 5 hatched out of 7.
,, (No. 2), 6 ,, ,, 7.
,, (No. 3), 4 ,, ,, 7.

All the larvae were making slow progress.

Peptone broth. Tube (No. 1), 5 hatched out of 6 (2 larvae dead); slight progress.
,, (No. 2), 4 ,, ,, 6; slight progress.
,, (No. 3), 2 ,, ,, 5; very slight progress.
,, (No. 4), 2 ,, ,, 6; little, if any, progress.

On the 8th day from start:

Boiled distilled water. Larva still in first instar.

Peptone water. No change, the larvae still in first instar.

Beef broth. A few more eggs had hatched; the numbers are now:

Tube (No. 1), 6 hatched out of 7.
,, (No. 2), 7 ,, ,, 7.
,, (No. 3), 4 ,, ,, 7.

All the larvae show slight growth, most of them being in the second instar, but one has not passed its first moult.

Peptone broth. The living larvae are now:

Tube (No. 1), 4 out of 6 eggs.
,, (No. 2), 4 ,, 6 ,,
,, (No. 3), 2 ,, 5 ,,
,, (No. 4), 4 ,, 6 ,,

Slight growth was apparent, but none had passed the first moult.

On the 11th day from start:

One of the *peptone water* tubes in which none of the eggs had hatched was inoculated with *B. coli*; by next morning (18 hours later) all the eggs in this tube had hatched.

In tube No. 4 of the *peptone broth* tubes sterility had broken down, and there was a slight bacterial infection. In this tube only were the larvae making any progress; in all the other peptone tubes the larvae were marking time.

Beef broth. In two tubes (Nos. 1 and 2), the larvae were growing steadily, if slowly; in No. 3 progress was very slow.

On the 14th day:

The larva which hatched in the boiled distilled water died in its first instar.

Peptone water. One of the two larvae which hatched in the sterile tube was dead. In the *B. coli* infected tube the larvae had already passed the second moult (about 18 hours after hatching). In the third tube, which remained sterile, the eggs were still unhatched.

Peptone broth. Tube (No. 3), one of the two larvae was dead, the other had moulted; (No. 2), 3 larvae were in the second and 1 in its third instar, all small and ill-grown. In tube No. 1 another larva had hatched, but one of those which had emerged previously was dead; of the living 2 were in the second and 2 in the first instar. In the infected tube (No. 4) the larvae were making slow but steady progress in their second and third instars.

Beef broth. Tubes Nos. 1 and 2 had become infected and the larvae were progressing.

15th day:

Distilled water. A second larva had emerged in this tube.

Tap water. Eggs still dormant.

Peptone water. In the *B. coli* infected tube all the larvae had reached their fourth instar and one had died.

To the sterile tube in which no eggs had hatched ·5 c.c. of sterile watery extract of brewers' yeast was added. (Without result.)

Peptone broth. Tube (No. 1), the two first instar larvae were taken to test if change to a different media would affect growth. A note will be found at the end of this experiment (page 511), both the second instar larvae were dead; (Nos. 2 and 3), no change; (No. 4), the infected tube, the larvae were making slow progress.

Beef broth. The larvae in the infected tubes (Nos. 1 and 2) progress steadily; in No. 3 the progress is scarcely perceptible.

19th day:

Distilled water. The second larva which hatched was also dead.

Tap water. None of the eggs had hatched.

Peptone water. In the tube infected with *B. coli* all the larvae reached full growth and then died.

Of the larvae which hatched out in the sterile tube, 1 had died; the living ones made no progress.

The eggs in the tube to which sterile yeast extract was added had not hatched.

Peptone broth. (No. 1), no living larvae remained; (Nos. 2 and 3), the larvae all dead; (No. 4), growth was variable, the larvae which hatched prior to infection were ahead of the others.

Beef broth. Larvae in the sterile tube were making very slow progress; in the infected tubes they were growing steadily, though not rapidly.

22nd day:

Distilled water and *Tap water.* No change.

Peptone water. In the sterile tube the larvae made no progress. The yeast extract did not cause the eggs to hatch in the third tube; this tube was inoculated with a living yeast *and the eggs hatched during the night.*

24th day:

Tap water. The eggs lay dormant in this tube for 24 days; 1 c.c. of an autolyzed extract of brewers' yeast was added (about 20 %); the eggs hatched within 15 minutes; the larvae, however, died.

Quantities, up to about 10 %, of this sterile extract were added to the sterile tubes of peptone water and peptone broth in which unhatched eggs were lying, but no further larvae emerged, and the larvae which had already hatched in these tubes did not respond by growing.

28th day:

Peptone water tube (in which the eggs hatched on the 22nd day following an inoculation with a living yeast). The larvae showed slow and varied growth; they eventually died without pupating—the growth of yeast in the tube being presumably too feeble to supply sufficient nutrition.

The larvae in the sterile tube of peptone water to which sterile yeast extract had been added, lived on for a long while, making little or no progress. A number were still living 95 days after the start of the experiment, and one lingered on until the 113th day, when it was still in its second instar.

Beef broth. The larvae in the sterile tube progressed very slowly, sterility being maintained as shown by the sub-culture tests made at intervals. After 50 days a few adults were reared; these emerged at intervals over a period of 10 or 12 days, one, which fell into the broth and was drowned, was transferred with a platinum loop to a tube of sterile broth, but no growth ensued. A living specimen was fed on a carefully cleansed patch of skin on the arm, producing the same quickly passing reaction mark as do normally bred *S. fasciata* when they bite this individual. This observation suggests that Schaudin's opinion that the reaction following the bite of the mosquito is due to a ferment is possibly incorrect. An attempt to feed another specimen, reared subsequently under sterile conditions, on the arm of a lady, who showed a very marked reaction to the bites of *S. fasciata*, failed as the insect died without biting, although tested on several successive days.

Result of test carried out on larvae taken from Tube No. 1 *peptone broth on the* 15*th day.*

Two larvae were withdrawn by Pasteur pipette on the 15th day from one of the peptone broth tubes which had remained sterile.

One of these larvae was transferred to a tube of broth made from dried and powdered insects (mostly house flies), the other to a tube of water, in which horse dung had been steeped. Both these tubes had been autoclaved. The plan was to test if a change of diet under sterile conditions would affect growth. The larvae at first made no progress, but after 48 hours they began to show signs of slow growth, which continued for 30 to 40 days, by which time the larvae were little more than half grown. They then marked time during a period of 10 or 12 days and died without making any further progress.

EXPERIMENT XII.

This test was carried out with a view to deciding if sterilized media of a kind more likely to be encountered in nature than those prepared for bacteriological experiments would allow of the larvae developing.

For this purpose tubes of water in which horse dung had been steeped for 48 hours and of a broth made in hot water from dead insects were autoclaved. Both media when tested under unsterilized conditions had been found to afford excellent breeding results.

Eggs sterilized by Method *b* were transferred to the tubes by Pasteur pipette.

Insect broth. No eggs hatched within 18 hours, but a few larvae were out in tubes Nos. 1 and 2 after 48 hours. None of the eggs had hatched in tube No. 3. One or two larvae were out in this tube also, after 72 hours. It was significant, however, that a small mould was observed on a group of floating eggs in tube No. 3. (Subsequent experiments showed that the growth of a mould acted as a stimulant, causing eggs to hatch.)

During 5 days the larvae made slow but steady growth in tubes 1 and 2, but made no perceptible progress in No. 3.

Tests showed that sterility had broken down in tubes 1 and 2, a feebly growing species of bacteria being present when tested after 8 days.

The development of the insects was very slow, taking about 50 days. In tube No. 3 a larva was still living, being only in its third skin after a period of 65 days. This larva died subsequently.

There was some mortality in the late larval stage, but a few adults were reared in each of tubes 1 and 2.

Manure water. No eggs hatched within 18 hours.

A few larvae were out in one tube within 48 hours.

Larvae were out in all tubes within 72 hours.

During 5 days the larvae made slow but steady growth in all the tubes, and were in either the close of the second instar or the beginning of the third. Progress was slower in one tube than the others, but tests proved that all the tubes were infected with some feebly growing bacteria. The larvae lived some 40 or 50 days, eventually reaching the fourth larval instar, but all died without pupating.

EXPERIMENT XIII.

A number of further trials were made, using insect broth and manure water. Eggs were transferred to 12 tubes of the former and 11 of the latter; in a few of these tubes sterility was maintained and some were used in Experiment XX.

In the majority of cases the eggs hatched in consequence of the development of moulds, or showed a feeble slow-growing bacillus. The results in the sterile tubes showed that very slow or no progress took place in them. The larvae which succeeded in growing finally died before their full size was attained and there was a suspicion in these cases of some feebly growing bacillus which had escaped the sub-culture test. In the more numerous instances where the presence of the feeble infection was proved the larvae seldom completed their development.

EXPERIMENT XIV.

Use of killed culture of B. coli.

Eggs placed in tubes containing broth cultures of *B. coli*, killed by heat, failed to hatch within a period of 5 days. A larva was transferred from a sterile tube in another experiment. This larva lived for a period of over a month, but failed to make any progress. The tube was finally inoculated from a living *B. coli* culture when the larva died. This result has followed in a number of instances when the sterile conditions in which larvae have been living for a long period are broken down. In some instances no doubt this has been due to the violent character of the growth, with which two or three small larvae are quite unable to cope, but, apart from this, it would seem that some adjustment to the sterile conditions has occurred which cannot easily be reversed. In contrast to this, is the fact that larvae which are only starved by keeping them short of food in an unsterilized condition, are quite able to take advantage of an influx of bacteria, rapid development following immediately.

EXPERIMENT XV.

Development of larvae on a diet of Staphylococcus aureus.

(Note also tube No. 7, Experiment IX.)

Eggs sterilized by Method *b* were transferred by pipette into two tubes of peptone broth. One tube was inoculated from a *B. coli* culture, the other from one of *S. aureus*.

Growth of the bacteria followed immediately in the *aureus* tube and the eggs hatched within a few hours of inoculation.

In the *B. coli* tube the inoculation failed; only 1 egg hatched, and the larvae died within 3 days. On the 4th day the tube was again inoculated from the same culture of *B. coli*; by the following morning it was observed that the second inoculation had been effective and that the eggs had hatched.

The larvae in both tubes grew rapidly and produced adults within 8 to 15 days.

EXPERIMENT XVI.

Question of the necessity of solid particles with the food, also note respecting the effect of past bacterial action on the eggs.

It was suggested by one of our colleagues that the mosquito larvae might be unable to obtain nourishment in a pure fluid, particulate matter being essential to their digestive processes. We therefore tried the effect of adding animal charcoal to some tubes and not to others. Eggs that had been kept moist from laying were used and sterilized by Method *b*. The theory here was to allow of bacterial action on the outer surface of the eggs up to the moment of their sterilization. Apparently, on the showing of this one test, the theory has some basis as the result showed more eggs hatching under sterile conditions than usual.

The eggs were transferred to tubes of peptone water, peptone broth, pure beef broth, 2 % and 3 % solutions of an autolyzed extract of brewers' yeast in distilled water. To the yeast extract tubes a small quantity of animal charcoal was added prior to their sterilization.

Eggs hatched in some of the tubes within an hour and many more within two hours; such rapidity suggests past rather than present bacterial action, as even if the tubes were infected at the placing of the eggs in them, there was little time for growth, whereas all the experiments suggest that it is the vigorous bacterial action of a maximum infection which counts in causing hatching.

After 60 hours the following had emerged:

Peptone water	2 out of 16.	Sterile.
	0 ,, 6.	,,
Beef broth	all ,, 18.	Infected.
	4 ,, 11.	Sterile.
Peptone broth	1 ,, 17.	Sterile; larva dead.
	7 ,, 17	,,
2 % solution yeast extract	several[1].	,,
	,,	,,
3 % solution yeast extract	4 tubes, a few in each tube.	

It was noted that all the larvae in the yeast extract tubes containing charcoal were making progress, the dark line of their guts showing that the charcoal was being swallowed.

After 84 hours:

Peptone water	3 larvae from 16 eggs.	Sterile; no progress.
	0 ,, 6 ,,	
Beef broth	all ,, 18 ,,	Rapid progress of larvae; gas production by bacterial growth.
	6 ,, 11 ,,	Very slight progress of larvae; still sterile.
Peptone broth	1 larva (dead) 17 ,,	Sterile.
	9 larvae from 17 ,,	Very slight progress of larvae; still sterile.
2 % solution yeast extract	4 larvae from about 15.	3 passed first moult.
	4 ,, ,, 15.	2 ,, ,,
3 % solution yeast extract	1 ,, ,, 15.	Has passed first moult.
	4 ,, ,, 15.	Have ,, ,,
	3 ,, ,, 15.	1 has passed first moult.
	5 ,, ,, 15.	3 have passed first moult.

With one exception the broth and peptone tubes passed out of the experiment owing to the larvae dying after a protracted period of minimal progress, or owing to sterility breaking down in the tubes, followed by the hatching of the dormant eggs and rapid development of the larvae.

Beef broth. In one tube of beef broth sterility was maintained; on the 41*st day* there were 4 living larvae in the third instar; on the 76*th day* tests showed that the tube was still sterile, some of the larvae having advanced to the fourth instar. On the 90*th day* 2 adults were reared; a small quantity of broth from the tube, together with one of the adult mosquitoes, was introduced into a tube of peptone broth which remained sterile. The original tube was infected with *B. coli* to see if any eggs were lying dormant; none hatched, so a few unsterilized eggs were added to test the nutritive properties of the broth after infection. Larvae emerged from the eggs the same day, by the 8th day nearly all had passed the third moult.

Yeast extract solution. Eggs were transferred after sterilization to a few tubes of 3 % solution of an autolyzed extract of brewers' yeast; no charcoal being added, larvae hatched in some of these tubes, but made still slower progress than did those with charcoal, behaving as did the larvae in sterile peptone water or peptone broth, generally dying in their first instar and but very exceptionally surviving to the third.

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[1] The presence of animal charcoal in the tubes rendered an exact count impossible.

2 % *solution of yeast extract with charcoal added.* After 50 days in one tube (No. 30) there were several larvae in the third instar and at least one in the fourth; 1 had pupated but the pupa died. All the larvae were lank and slender and had failed to develop the fat bodies which, towards the close of the larval life, become rather obvious owing to the opaque white appearance that they give to the larvae.

The second tube (No. 31) contained 4 larvae in their fourth instar, all deficient in fat deposits.

One tube (No. 32) contained a single weakly looking fourth instar larva; the remainder were dead.

Another (No. 33) contained 4 fourth instar larvae, all deficient in fat.

In tube No. 34 none of the larvae had got past the third skin. By the 56th day the larvae in tubes 31 and 32 were dead.

One larva was living in tube 30 on the 76th day, but died before the 95th day. In tube No. 33 two larvae lived until the 76th day and one until the 123rd day, when it died. In tube No. 34 two larvae lived until the 76th day; between the 76th and 95th days several larvae hatched from dormant eggs in this tube. Tests showed that sterility was still maintained; by the 107th day all the recently emerged larvae were dead except one, and this had passed the first moult; two died between the 107th and 123rd day. Between the 123rd and 143rd day moulds developed in the tube and the last surviving larva died.

EXPERIMENT XVII.

A further series of tubes containing a 3 % solution of autolyzed extract of brewers' yeast in distilled water, some with and others without charcoal, were prepared.

23 *days from start.*

No. 51,	sterile,	larvae.	Slender in second or third instar.
No. 52	,,	,,	Slender in third or fourth instar.
No. 53	,,	,,	Great disparity of growth in all instars from first to fourth skin; all slender.
No. 54	,,	,,	Slender in second or third instar.
No. 55	,,	,,	Great disparity of growth in all instars from first to fourth skin; all slender.

Tubes, *with charcoal*, 56 and 57.

No. 56, a mould grew, the larvae had grown steadily and were in fourth instar.

No. 57, sterile larvae show slow but healthy growth, are rather more robust than those in the tubes without charcoal, but are only in second and third skin.

40 *days from start.*

Nos. 51 to 55 *without charcoal.* No appreciable progress in the last 17 days.

Nos. 56 and 57 *with charcoal.*

No. 56, larvae have all died without pupating.

No. 57, no appreciable progress.

56 *days from start.*

Nos. 51 to 55 *without charcoal.*

No. 51, sterile. Most of the larvae in their third instar; one or two possibly dwarfed in fourth instar.

No. 52, sterile. Only 2 larvae living, 1 in third instar, 1 in fourth instar.

No. 53, a mould present; 1 ♀ reared, larvae in second, third and fourth instars, still living.

No. 54, sterile. All in third instar.

No. 55, sterile. 2 larvae in fourth instar; 1 pupa.

With charcoal.

No. 57, all the larvae now in third instar.

78 *days from start.*

Without charcoal.

No. 51, apparently sterile; little if any change.

No. 52, ,, ,, ,, ,,

No. 53, mould present; most are in fourth instar.

No. 54, apparently sterile; little if any change.

No. 55, apparently sterile; 1 adult, 1 third or dwarfed fourth instar larva; 1 larva dead.

With charcoal.

No. 57, sterile; slight if any change.

90 *days from start.*

Without charcoal.

No. 51, sterile; no noticeable change.

No. 52, ,, ,, ,,

No. 53, mould has increased in size; only 1 larva living

No. 54, sterile; no noticeable change.

No. 55, ,, ,, ,,

With charcoal.

No. 57, sterile; slight progress.

106 *days from start.*

Without charcoal.

No. 51, sterile; no apparent change.

No. 52, sterile; dormant eggs have hatched; there are now 5 living larvae.

No. 53, all dead, the last surviving larva died on or about the 95th day.

No. 54, sterile; no noticeable change.

No. 55, ,, ,, ,,

With charcoal.

No. 57, sterile; only 1 larva now living.

126 *days from start.*

Without charcoal.

No. 51, sterile; little if any change; 3 larvae still living.

No. 52, sterile; 4 larvae living; those recently hatched have made slight progress.

No. 54, sterile; 2 larvae living; little if any change.

No. 55, sterile; 2 larvae living; 1 in fourth and 1 in second instar.

With charcoal.

No. 57, the last larva is now dead.

Evaporation had so reduced the quantity of fluid that boiled distilled water was added to tubes Nos. 51, 52, 54 and 55. Following the addition of the water a few eggs that had lain dormant in tubes Nos. 51, 54 and 55 hatched.

Nine days later, 135 *days from start.*

No. 51, it was found that all the larvae were dead.

No. 52, 1 of the 4 larvae died.

No. 54, only 1 of the 2 original larvae living; those that hatched out recently are dead.

No. 55, the recently hatched larvae have disappeared, possibly swallowed by the larger ones.

Tube No. 54 inoculated with a yeast (*S. cerevisiae*).

142 *days from start.*

No. 52, all the old larvae are dead, the last two died with their jaws entangled in a small tuft of hair. The three survivors are growing steadily. (Sterile.)

No. 54, this tube was inoculated with a yeast (*S. cerevisiae*); the surviving larva after one week looks large and stout in comparison with its dead companions.

No. 55, the surviving old larva is still active, two of those which hatched after the addition of fresh water are living and are now in second skin. (Sterile.)

152 *days from start.*

No. 52, moulds grew in the tube; the three last larvae died.

No. 54, the surviving larva pupated.

No. 55, the old larva is dead and also one of the recently hatched ones.

Tube No. 55 was inoculated with (*S. cerevisiae*).

168 *days from start.*

No. 54, the pupa died.

No. 55, an adult ♀ was reared from the surviving larva in this tube.

EXPERIMENT XVIII.

Killed B. coli *culture and manipulated milk.*

A tube of distilled water, to which washed *B. coli* had been added, was sterilized by heat, and a sterile larva introduced that had hatched out in peptone broth. After 13 days during which the larva made no progress, 1 c.c. of sterilized milk, the colloid particles of which were increased in size but not precipitated by a manipulation of its acidity, was added. No progress occurred within 3 days; the tube was inoculated from a tube of broth in which a certain species of bacterium had developed. (This culture was used because its speed of growth under the conditions of the experiment had allowed the larva in the tube to develop satisfactorily.) The larva at once commenced to grow, and pupated within 20 days, an adult emerging in due course.

Experiment XIX.

A number of tubes were prepared and sterilized containing appropriate quantities of the following in distilled water; sterile filtrate from a culture of *B. coli* with white of egg, treated so as to form a fine precipitate; filtrate of *B. coli* with milk in which the casein particles had been increased in size; and *B. coli* filtrate without any addition.

Larvae were taken from a sterile tube of peptone water in which they had hatched 16 days previously; they were still in the first instar, having made barely perceptible progress during 16 days. Slight progress followed over a protracted period in the tubes (containing white of egg and milk) which remained sterile, but in all cases it came to a standstill when the larvae were still small, in the second or third skin, after which they pined away and died. In one or two instances sterility broke down and was generally followed by a sudden spurt of growth. In most cases, however, the growth of bacteria proved over-violent and killed the insects ere they had time to develop.

Experiment XX.

The treated milk was also added to tubes of yeast extract solution, peptone water and manure water, in which eggs had hatched and the larvae were unable to develop owing to the sterile conditions. In no case did the addition of the milk produce any change in the scarcely perceptible progress that the larvae made. Sterile filtrate of *B. coli* in the proportion of about 1 in 10 was added to these tubes after the lapse of 10 or 12 days, and also to sterile tubes of insect broth and 3 % yeast extract solution. During 5 days no change resulted from the addition of the filtrate, the scarcely perceptible growth that was taking place in some only of the tubes was not appreciably quickened, in other cases when the larvae had already arrived at the marking time phase no progress was induced.

The following notes refer to some of the tubes mentioned above.

Insect broth. A larva that had hatched under sterile conditions (see Experiment XIII) lived in the tube for 34 days when it was still in the first skin; 1 in 7 of *B. coli* filtrate was added. No change took place in 5 days; then the tube was inoculated with *B. coli*; eggs which had been lying dormant in the tube for 39 days hatched as a result. Rapid growth of the larvae followed, but it is not possible to say definitely if the larva which had been marking time for so long was one of those which developed to adult stage within 10 days; 4 had pupated and 3 ♂♂ and 1 ♀ emerged in due course.

Peptone water. The two or three larvae which hatched from the eggs pipetted into the tube made no progress during 18 days; the treated milk was added—the larvae made no progress and died out within three weeks. Eggs had been lying dormant in this tube for 39 days. The tube was inoculated with *B. coli* and these eggs hatched within a few hours. The larvae commenced to make rapid progress, but their history was not followed up.

Yeast extract in distilled water. Three or four larvae hatched from the eggs placed in this tube. They made no perceptible progress during a week; treated milk was added to the tube. After 20 days two of the larvae had passed the first moult; 76 days after the addition of the milk the larvae were still in the second or third instar, and one had failed to complete its moult satisfactorily. Three larvae were still living on the 88th day after the addition of the milk; two were living in the same stage after 104 days and one after 124 days. Sterile water was then added to the tube to replace evaporation and the tube was inoculated from a *B. coli* culture; the violence of the growth, however, killed the larvae. (Eggs were added to the tube; they hatched almost immediately and the larvae were killed off within a day, showing that the death of the larvae which had lingered on for so long a time was not in all probability connected with their age.)

Various egg hatching and larva feeding tests.

Experiment XXI.

Sterile tubes prepared of filtrate from a *B. coli* culture; *B. coli* filtrate and precipitated white of egg; *B. coli* filtrate and milk in which the casein particles had been enlarged; autolyzed yeast extract in distilled water; peptone water; and distilled water.

Eggs sterilized by Method *b* were pipetted into each tube.

No. of eggs hatched in	Tubes	2½ hours	20 hours	48 hours	96 hours
Filtrate of *B. coli* culture	1	2	3	All hatched*	All* larvae growing rapidly
	1	1	1	All hatched*	All* larvae growing rapidly
	1	0	1	All hatched*	All* larvae growing rapidly
	1	0	0	1	All* larvae growing rapidly
	1	0	0	0	2, hatching became general and a mould appeared
	1	0	0*	Used for special test, see Experiment IV, page 503.	
Filtrate of *B. coli* and precipitated white of egg	1	0	1	All hatched*	Larvae growing rapidly
	1	0	0	All hatched*	Larvae growing rapidly
Filtrate of *B. coli* and treated milk	1	0	1	All hatched*	Larvae growing rapidly
Autolyzed extract of brewers' yeast, in distilled water	1	0	0	2	No change
	1	0	0	0	
	1	0	0	0	
Peptone water	1	1	1	1	No change
	1	0	0	0	1
Distilled water	1	1	5	6	10
	1	1	3	3	5
	1	0	2	Used in a special test; see Experiment IV, page 503.	
	1	0	2		
	1	0	2		
	1	0	1		

* Tested for sterility—all proved to be infected.

After 120 *hours.* The larvae in all the starred (infected) tubes continued to grow rapidly: in the filtrate tube in which a mould appeared, all the eggs hatched but the larvae made no progress.

Films from the filtrate tubes in which sterility broke down stained by Gram's method showed a mixed infection of bacteria other than *B. coli.* One tube showed sporing forms. The results were consistent with the explanation that the tubes were infected by organisms on the eggs, some of which had escaped the sterilizing process.

Of the two peptone water tubes all the eggs in one tube hatched; a mould was noticed developing in the tube. The larvae made little if any progress during a period of 20 days, the mould gradually increasing at the surface of the fluid. The larvae were all dead within a month of hatching. 1 c.c. of the sterile filtrate of *B. coli* was added to the remaining peptone water tube and the two tubes of distilled water. Within 4 days all the eggs hatched in the peptone water tube, but it was evident that sterility had broken down. The larvae made rapid progress, but failed in their race with the bacteria—the fluid in the tube becoming very foul—and the larvae died.

Sterility also broke down in the two tubes of distilled water, to which the filtrate was added. It is quite possible that it had broken down at the time when the eggs began to hatch, but as the tube was not tested the fact was not apparent until the filtrate was added. The larvae made rapid progress at first, but it soon appeared that they had exhausted the food supply as, although they continued healthy, no progress was made. The larvae gradually died off—a few survivors lingered on until the 88th day; they were then small and undersized in the third or fourth instar. Moulds were developing in both tubes and the death of the larvae followed in a few days. Concerning the filtrate tubes to which precipitated white of egg and treated milk were added nothing of interest remains to be said; all were infected with a species of bacterium that required cool conditions—60° to 65° F.—for its favourable development. The larvae grew rapidly at first but slowed down after a few days; after the 8th day they were still in the third instar. Those in the tube to which milk was added attained the fourth instar by the 12th day and then died. The larvae in one of the white of egg and filtrate tubes developed slowly and 2 adults were reared within a month, but a mould had developed in the tube, and the remaining larva died. In the other tube a mould also developed and all died as larvae.

3 % *Yeast extract solution.* Nos. 106 and 107. No larvae hatched within 96 hours; but one was out in each tube within 120 hours; these larvae died within 2 or 3 days. In one tube no more eggs hatched within 10 days; in the other a few larvae emerged and died. On the 12th day both tubes were inoculated from a culture of a yeast; the eggs hatched during the night, in one tube they all died, but in the other a few of the larvae made rapid progress. Three ♂ specimens were reared within 18 days of the inoculation and three ♀♀ followed a week or so later.

2 % *Yeast extract solution with charcoal.* One tube; within 24 hours 6 or 7 larvae had hatched, the larvae making slight if any progress within 3 days of hatching; within the next few days moulds commenced to grow. By the 7th day after hatching a few larvae had passed their first moult. Some of the larvae attained to the fourth instar within 20 days of hatching, but the growth of moulds had made

considerable progress by this time, and the larvae died within 2 or 3 days. *Infection by bacteria in addition to the moulds was probable.*

EXPERIMENT XXII.

Planned to contrast the effect, if any, on hatching of *B. coli* filtrate, beef broth and distilled water. It failed owing to the number of tubes in which sterility broke down. The experiment, however, affords evidence relative to the effect of the growth of moulds and the ability of the larvae to develop on a diet of living yeast cells.

To each of 9 tubes containing 6 c.c. of sterile distilled water, was added 1 c.c. of filtrate from a culture of *B. coli*.

To each of 6 tubes was added 1 c.c. of plain beef broth.

Eight tubes contained the distilled water only.

Eggs were sterilized by Method *c* and from 12 to 20 pipetted into each tube.

After 24 hours.

Filtrate and water. 7 of the tubes have at least 20 eggs; 2 have only 12 in them. In all the tubes there are one or more larvae swimming. In most of the tubes there are 5 or 6 larvae; in one 8 or 9, and in one a dozen.

Beef broth. Four of the tubes had 12 to 18 eggs; two had at least 20 eggs in them. The hatchings were: 2 : 1 : 2 : 1 : 8 : 2.

Distilled water. Six of the tubes had from 12 to 20 eggs; two had 20. The hatchings were: 1 : 1 : 0 : 0 : 0 : 2 : 0 : 0.

After 4 days.

Filtrate and water. In one tube (No. 9) there was a bacterial growth; the water was cloudy; all the eggs had hatched and the larvae showed vigorous progress. In all the remaining tubes, though hatching was general, larval progress was not evident; there was no bacterial growth, but moulds had commenced to grow in the tubes.

Beef broth and water. In two tubes (2 and 6) bacterial growth started; in two (1 and 5) moulds grew, while two (3 and 4) remained sterile. Hatching was general, but not complete in those tubes which were not infected by bacteria or moulds; larval growth was rapid only in those containing bacteria.

Distilled water. In two of the tubes two or three eggs hatched; in three one hatched, while in three no larvae emerged.

Subsequent history.

Filtrate and water. Nine tubes numbered 1 to 9.

No. 1 had a double infection of bacteria and moulds; one undersized ♂ bred after 35 or 40 days, remainder died.

No. 2, white surface moulds gradually increased, and the larvae gradually died off; 1 lived 60 days, and died in the third instar.

No. 3, dark surface moulds grew gradually; 1 larva lived 53 days but died in the third instar.

No. 4, grey surface moulds grew gradually; none of the larvae passed the second moult—all died within 50 days.

No. 5, the mould in this tube was more vigorous; it formed flocculent tufts just beneath the surface. The last larva to die was in its third instar. None lived beyond 50 days.

No. 6, white surface moulds grew gradually; 3 larvae were living on the 53rd day; the last survivor died in the third instar within 60 days.

No. 7, in addition to floating moulds, this tube had a heavy bacterial growth in it. 2 fourth instar and 1 second instar larvae were living on the 69th day, but all died without reaching pupal stage.

No. 8, small globular moulds developed at the bottom of the tube; all the larvae died in the first or second instar early in the course of the experiment.

No. 9, this tube had an obvious infection, a surface scum formed, but there was no trace of masses of mould, as in the other tubes; 5 larvae lingered in the second and third instars until the 53rd day, but all died out by the 69th.

Beef broth and water.

No. 1, a white surface mould; all larvae died in first and second skin.

No. 2, a weak, double bacterial infection; larval progress slight; several larvae in second and third skin were living on 53rd day; by the 106th day 1 larva was still living and a sub-culture in broth gave no result, the infection being presumably too slight. 1 c.c. of broth was added to the tube and subsequently ·5 c.c. from the tube was added to sub-culture broth tube. Result: infected. A larva hatched from a dormant egg within two hours of the addition of broth, presumably as the result of increased bacterial growth consequent upon the addition of fresh nutritive material.

No. 3, this tube remained sterile to all the tests applied; 1 larva reached second and 1 the third skin by the 53rd day; both died subsequently.

No. 4, this tube remained sterile; 5 larvae were still living in the first instar on the 21st day; on the 28th day the larvae showed little if any progress. A mass of living yeast cells (*S. cerevisiae*), lifted from a wort agar slope, was added to the tube; several dormant eggs hatched within a few minutes. The larvae all grew rapidly and cleared the water, which became cloudy when the yeast cells were introduced, within 5 days. A fresh loopful of the yeast cells was added. In 2 days' time the water was again clear, and a fresh loopful was given. On the 13th day most of the larvae were in their fourth skin and one had pupated. Another loopful of the yeast cells was given. A week later a male was reared and a loopful of yeast cells added. A second adult, a ♀, was reared on the 25th day after infection. A further loopful of the yeast cells was given; by the 32nd day 3 adults had been reared. There were 2 pupae and 4 fully developed larvae; further yeast cells were given. In all 8 adults were reared in 40 days. The period taken in this instance was due to the lapse of time between the meals and the small allowance of food given at any one time; had a larger mass been given, not above one-third of the time would have been taken.

No. 5, several very small moulds grew at the bottom of the tube. The larvae made slight progress; the moulds did not develop, and apparently died out. The larvae made very slight progress, but by the 40th day after hatching none had passed the second instar. Some of the larvae died, others made slow progress. After 100 days there were 2 living, 1 in the third and 1 in the fourth instar. Tested and found sterile on the 100th day.

No. 6, this tube developed a vigorous bacterial growth; the larvae made rapid progress, but all died in the second or third skin.

Distilled water. Of the eight tubes to which eggs were added only one showed infection by the tests used; on the 20th day of the experiment the position was as follows:

No. 1, 2 eggs hatched, 1 larva dead, 1 living, both in first skin.

No. 2, none hatched.

No. 3, 3 hatched, 1 larva dead, 2 living, all in first skin.

No. 4, 6 hatched, all dead in first skin.

No. 5, none hatched.

No. 6, 5 hatched, 4 dead, 1 living, all in first skin.

No. 7, many hatched, probably all, larvae all dead but one; none past first skin, infected by mould.

No. 8, 1 hatched; larva living in its first skin.

All the living larvae died within a week or two; the tubes containing dormant eggs were used for the under-noted experiment.

On the 28th day of experiment a loopful of living yeast cells (*S. cerevisiae*) was added to *tube No.* 1. Eggs which had been lying dormant for 28 days hatched within 5 minutes; rapid growth ensued and from time to time a fresh loopful of the yeast cells was added. 5 adults were reared and one specimen died in pupal stage. The time taken was from 30 to 50 days; the quantity of food given at five-day or weekly intervals—was a small mass of cells scraped from a wort agar slope with a platinum loop—only allowing of development by fits and starts.

Tubes Nos. 2, 3, 4, 5, 6 and 8 were infected by adding ·5 c.c. of a yeast culture; larvae hatched from dormant eggs in most of the tubes within 5 minutes; others followed rapidly.

All the larvae made rapid progress except those in tube No. 5; here moulds started to grow rapidly. Within a week there was only one larva living, and this one also died within a day or two.

A small quantity, usually about ·2 or ·3 c.c. of the yeast culture, was added at intervals of several days or a week; the point it was wished to establish being not the speed with which the larvae could develop on a diet of yeast cells, but the fact that they marked time during the intervals and spurted when the food was given. In spite of the rather crowded condition of the larvae in 6 or 7 c.c. of fluid, they remained healthy, except in tube No. 4 where moulds developed. In this tube all the larvae died. The result showed as anticipated the entire dependence of the larvae on the added yeast culture, their speed of progress being conditioned by the length of the periods between each addition to the tubes. In tube No. 2, 6 adults were reared, in tube No. 3, 6, and 6 individuals in tube No. 6; while in tube No. 8 which contained more larvae to start with 9 adults were reared.

Experiment XXIII.

The increasing proportions of tubes in which sterilization broke down either from sporing species of bacteria or moulds, suggested that the contamination of the eggs laid in the breeding-pan was very gross. Steps were therefore taken to obtain eggs laid under more cleanly conditions and less likely to be heavily infected with sporing bacteria and moulds (the method adopted is described on pp. 488–9).

Eggs were used that had had time to incubate, but had not dried; these were well washed in three changes of clean tap water, put on to clean filter paper and then dried; they were subsequently sterilized by washing in weak ·5 % lysol, then in 2 %, then in 1 %, and then in sterilized distilled water; 9 or 10 eggs were transferred by Pasteur pipette to the following tubes:

Distilled water, Nos. 1 to 6.

Distilled water with 1 c.c. of sterile *B. coli* filtrate (= 1 in 6), Nos. 7 to 12.

Beef broth (without peptone or salt), Nos. 13 to 18.

Beef broth with 1 c.c. of sterile *B. coli* filtrate (= 1 in 6), Nos. 19 to 24.

Media	No. of tube	Hatching after 24 hours	After 5 days	Remarks after 5 days
Distilled water	1	0	0	The single larva which emerged did not grow. In one or two other tubes eggs uncapped, but the larvae died without quitting the shell.
	2	0	0	
	3	1	1	
	4	0	0	
	5	0	0	
	6	0	0	
Distilled water and filtrate of *B. coli* (= D.W. 6 c.c., F. 1 c.c.)	7	0	0	Sterility broke down after 24 hours in Nos. 9, 10 and 11, and the larvae which emerged made rapid progress.
	8	0	0	
	9	2	All*	
	10	0	All*	
	11	3	All*	
	12	0	3	
Beef broth without salt or peptone	13	2	All*	Heavy growth of bacteria, larvae already in fourth instar.
	14	1	1	Larva makes no progress.
	15	0	1	Larva makes scarcely any progress.
	16	0	1	Larva makes no progress.
	17	0	0	
	18	1	1	Larva makes slight progress.
Beef broth with filtrate of *B. coli* added (= B.B. 6 c.c., F. 1 c.c.)	19	0	0	
	20	0	2	Larvae show no progress.
	21	0	1	Larva shows no progress.
	22	0	7*	Heavy bacterial growth, larvae are already in fourth instar; remaining eggs failed to hatch.
	23	0	0	
	24	1	1	Larva shows no progress.

On the 6th day tube No. 15 was inoculated from No. 13; all the eggs had hatched by the following morning.

After the 7th day.

Distilled water and Filtrate. There was a distinct difference as regards the progress of the larvae in the infected tubes 9, 10 and 11; while the larvae in 9 and 11 were on a parity as to size, those in 10 were slightly but definitely smaller. Subcultures from these tubes show that, while the bacteria infecting 9 and 11 are closely allied, probably the same species, the infection of 10 differs in that it shows a much less vigorously growing species. The interesting point is that, although the difference in larval progress is slight, the divergence between the vigour of bacterial growth is extreme.

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* Infected tubes.

After the 13th day.

Distilled water. Tubes 1 to 6. The only larva which emerged has made no progress and is now very feeble.

Distilled water and Filtrate. The larvae in tubes 9, 10 and 11 are all of full size, but none of them have as yet pupated. In No. 12 three more eggs have hatched, none of the 6 larvae have as yet passed their first moult.

Beef broth. In tube No. 13 all the larvae have been killed by the vigour of the bacterial growth. In No. 14, 3 more eggs have hatched. In No. 15 the larvae are now either just approaching or past the second moult. This tube was passed on to a friend and kept at a lower temperature than the others—60° F. instead of 75° F. In No. 16 another egg has hatched, neither of the two has made any progress and one has died. In No. 17 one egg has hatched. In No. 18, 5 more eggs have hatched, but only the larva which emerged 12 days ago has made any progress and this one has not yet moulted.

Beef broth and Filtrate. No. 20, one of the two larvae is dead; their progress since hatching is only just perceptible.

No. 21, the larva has died without moulting.

No. 22 (infected), 7 adults have been bred, 6 ♂♂ and 1 ♀; 1 living pupa remains.

No. 23, 1 egg has hatched.

No. 24, the larva has died without moulting.

After the 21st day.

Distilled water. 1 to 6; the larva which emerged in tube 3 died in its first instar.

Distilled water and Filtrate. In tubes 9 and 10 the larvae are marking time in their fourth instar. In No. 11, 2 ♂♂ have been bred, the remaining larvae, of full length, are marking time. In No. 12 the 6 larvae make scarcely perceptible progress.

Beef broth. In tubes Nos. 14, 16, 17 and 18 the larvae are making very slow progress; a few are approaching, and one or two have passed their first moult. In tube No. 15, inoculated from No. 13 and passed on to a friend, the larvae made comparatively slow progress, presumably owing to the low temperature at which the tube was kept; apparently the vigour of the bacterial growth was the cause of their destruction.

Beef broth and Filtrate. In tube No. 19 one egg uncapped, but the larva was either already dead or died immediately afterwards, as it failed to get clear of the eggshell.

No. 20, 2 more eggs have hatched. The survivor of the first two larvae is now in its second instar.

No. 22 (infected), another adult was reared (8 in all).

No. 23, the larva from the only egg which hatched is now in its second instar.

No. 24, another egg has hatched; the larva is now in its second instar.

After 28 days.

Distilled water. No change; eggs still dormant.

Distilled water and Filtrate. Nos. 9 and 10, the larvae still marking time.

No. 11, another adult reared; some of the larvae have died, only 1 is now living.

No. 12, only 4 of the 6 larvae are living; all the larvae are still in their first instar.

Tubes Nos. 7 and 8 were used in a demonstration at a meeting of the Entomological Society of London. To No. 7 was added a small mass of yeast cells from a wort agar culture of *S. cerevisiae* and to No. 8 a small mass from a culture of *B. coli*. The eggs in No. 7 hatched within a few minutes, those in No. 8 during the following night.

Beef broth. No. 14, 1 larva in first and 3 in the second instar.

No. 16, 3 larvae in first and 2 in second instar.

No. 17, 4 larvae in first and 1 in the second instar.

No. 18, 1 larva has died; 4 are still in their first and 1 is in its second instar.

Beef broth and Filtrate. No. 19, no change.

No. 20, another egg has hatched; 3 larvae are in their first and 1 in its third instar.

No. 21, no change.

No. 23, another egg has hatched; the first larva is still in its second instar.

No. 24, 2 more eggs have hatched; the older larva is now in its third instar.

After the 33rd day.

Distilled water. No change.

Distilled water and Filtrate. Nos. 7 and 8, there is a marked difference in the growth of the larvae in these two tubes. Approximately the same bulk of *B. coli* was put into No. 8 as there was of yeast cells into No. 7. The larvae in No. 7 have only reached the second and third instars while those in No. 8 are all either in the third or fourth instar. This difference can hardly be owing to a minute difference in mass of the bacteria or yeasts which were added; probably it is due to the medium (distilled water with a little filtrate of a broth culture of *B. coli* added) being better adapted for the multiplication of bacteria than the yeasts. The fourth instar larvae in tubes 9 and 10 and the remaining larva in tube 11 are all marking time, having presumably exhausted all the nutriment without fully developing their fat bodies. In tube 12 all the larvae, save one, died in their first or second instars; the survivor, still in its first instar, is only capable of feeble movement.

Beef broth. No. 14, 1 larva is dead.

No. 16, no noticeable change.

No. 17, two larvae have died, the survivors are 1 in second and 1 in third instar.

No. 18, no change.

Beef broth and Filtrate. Nos. 19 and 20, no change.

No. 21, the larva which was in its second instar is dead.

No. 23, no change.

No. 24, only 1 larva in its first instar is now living.

After 49 days.

Distilled water. No change.

Distilled water and Filtrate. No. 7, these larvae are now in their third and fourth instars.

No. 8, 4 adults have been reared (within 21 days); there still remain 1 pupa and 1 full-grown larva in the tube.

No. 9, all the larvae but one are dead.

No. 10, 1 adult has been reared; there are still 4 full-grown larvae in the tube.

No. 12, the only larva is dead.

Beef broth. No. 14, no change.

No. 16, 1 has died; the 3 survivors have made no appreciable progress.

No. 17, the larva in its second instar has died, but 2 more eggs have hatched; the remaining larva is still in its third instar.

No. 18, 2 of the larvae in first instar have died.

Beef broth and Filtrate. No. 19, no change.

No. 20, 1 of the larvae has died.

Nos. 21, 23 and 24, no change.

After 50 days.

Beef broth and Filtrate. Tube No. 19 was inoculated from a culture of *B. coli* on the 50th day; 8 or 9 eggs hatched within a few hours.

After 60 days.

Beef broth and Filtrate. About ·5 c.c. of a broth culture of *B. coli* was added to tube No. 21 on the 60th day. A number of dormant eggs hatched within 10 to 15 minutes.

After 69 days.

Distilled water. No change.

Distilled water and Filtrate. No. 7, 1 adult reared; 1 died in pupal stage; 3 fourth instar larvae still living. A slow growing surface mould is present.

No. 8, 2 more adults reared (6 in all).

Nos. 9, 10 and 12, no change.

Beef broth. No. 14, another larva has died; the 2 survivors appear very feeble.

No. 16, all the larvae are dead.

No. 17, 1 of the 3 larvae is dead.

No. 18, 1 of the 2 larvae is dead.

Beef broth and Filtrate. No. 19, 5 adults reared, 1 larva died when full grown.

(Note these eggs were dormant for 50 days and the adults were reared within 20 days of inoculation of the tube with *B. coli.*)

No. 20, 1 larva has died.

No. 21, the larvae which hatched in this tube after its inoculation with *B. coli* were full grown within 7 days.

No. 23, the second instar larva is dead; another egg has hatched.

No. 24, no change.

After 78 days.

Distilled water. No. 7, a second adult reared; 1 larva living, the others died in fourth instar.

No. 9, the last larva has died.

No. 10, a second adult reared; 3 living larvae remain.

No. 12, no change.

Beef broth. No. 14, both the larvae are dead.

No. 17, both the larvae are dead.

No. 18, the larva is dead.

Beef broth and Filtrate. No. 20, no change.

No. 21, 7 adults reared within 16 days of the hatching of the eggs.

No. 23, no change.

No. 24, the third instar larva is dead; only 1 of the younger larvae is living, it is still in its first instar.

After 87 days.

Distilled water. No change.

Distilled water and Filtrate. No. 7, 1 larva marking time; moulds are growing out of the mouths of the dead larvae.

Nos. 10 and 12, no change.

Beef broth. No change.

Beef broth and Filtrate. No. 20, only 1 first instar larva is now living.

No. 21, the last larva has died.

Nos. 23 and 24, no change.

After 100 days.

Distilled water. Tubes 4, 5 and 6 were used for the following experiment in conjunction with Experiment XXIV, page 530.

To tube No. 4 about 3 % of a living culture of *B. coli* was added.
,, No. 5 ,, 3 % of a sterile autolyzed extract of brewers' yeast.
,, No. 6 ,, 3 % of a killed culture (autoclaved) of *B. coli.*

Result: No. 4, 1 hatched within 2 minutes; 2 more within 5 minutes; 2 more within 10 minutes.
No. 5, 1 hatched within 15—20 minutes.
No. 6, 1 ,, ,, ,, ,,

After an hour and a half:
No. 4, 8 out of 10 eggs hatched.
No. 5, 1 ,, 9 ,, ,,
No. 6, 1 ,, 11 ,, ,,

A small quantity of living yeast cells from a wort agar culture was then transferred by platinum loop to tube No. 5 and the remaining 8 eggs hatched within 15 minutes.

About 3 % of a living broth culture of *B. coli* was added to No. 6, and 1 egg hatched within 3 minutes, another followed within 5 minutes and 4 more within 15 minutes = 6 out of 10.

After 110 *days.*

Distilled water. Nos. 1, 2 and 3, no change.

No. 4, larvae now in second instar.

No. 5, ,, ,, third and fourth instars.

No. 6, ,, ,, second and third instars.

The rapid growth of the larvae in tube No. 5 should be noticed in contrast to what happened with regard to tubes Nos. 7 and 8, distilled water and filtrate, after infection with yeasts and bacteria on the 28th day. Presumably this difference is due to the media in tube No. 7 having been unfavourable for the yeast.

Distilled water and Filtrate. No. 7, a further development of moulds; the larva is dead.

No. 10, all died as larvae with the exception of one which reached the pupal stage.

No. 12, an egg had hatched.

Beef broth. Nos. 14, 16, 17 and 18, all the larvae in these tubes are dead; possibly some living eggs remain dormant.

Beef broth and Filtrate. Nos. 20, 23 and 24, all the larvae are dead; possibly some living eggs are lying dormant.

After 134 *days.*

Distilled water. Nos. 1 and 3, small white moulds developed in the tubes.

Nos. 4, 5 and 6, want of nutriment checked development; a few larvae are living in their second and third instar but many are dead.

Distilled water and Filtrate. No. 10, another adult reared.

No. 12, another egg hatched; both the larvae which hatched after the 87th day and this one which hatched after the 110th day are in their first instar.

Beef broth. No larvae are living in these tubes, but it is possible that there may be dormant eggs.

Beef broth and Filtrate. In No. 23 an egg has hatched since the 110th day.

After 156 *days.*

Distilled water. Tube No. 2 was infected with *B. coli* without result: it was concluded that all the remaining eggs were dead.

Nos. 4, 5 and 6, 1 larva was living in No. 4 and 3 in No. 5; all were dead in No. 6.

Distilled water and Filtrate. No. 12, only 1 larva living.

Beef broth. No. 14, tested with *B. coli* for dormant eggs; none hatched.

No. 16, an egg had hatched since the 134th day.

Nos. 17 and 18, a little of a broth culture of *B. coli* was added; dormant eggs hatched in both tubes within 15 minutes.

Beef broth and Filtrate. The larva which hatched out of a dormant egg in No. 23 is dead.

An egg hatched in tube No. 20 after the 134th day.

After 203 *days.*

All the larvae are dead except the one in distilled water and filtrate No. 12; this is now in its fourth instar. One adult was reared in tube No. 5, distilled water.

The remaining tubes were infected with a yeast to see if any dormant eggs survived. Single eggs hatched in tubes No. 1 distilled water and No. 6 broth and filtrate. Further quantities of yeast cells were added to distilled water and filtrate tube No. 12 on the 204th day; the larva pupated on the 205th day and an adult ♂ was reared on the 208th day.

EXPERIMENT XXIV.

Planned to test further the effect of sterile cultures of bacteria and extracts of yeasts on dormant eggs.

Twenty-four tubes each containing 8 c.c. of distilled water were sterilized and a number of sterile eggs varying from 20 to 50 were introduced. After 5 days the tubes were examined, and a selection made of those in which only a few eggs had hatched.

The following were chosen:

No. 1,	about 50 eggs,	6 hatched;	all the larvae dead.
No. 2	,, 25 ,,	none ,,	
No. 3	,, 40 ,,	2 ,,	all the larvae dead.
No. 4	,, 30 ,,	1 ,,	larva dead.
No. 5	,, 50 ,,	4 ,,	1 larva living.
No. 6	,, 45 ,,	3 ,,	all the larvae dead.
No. 7	,, 30 ,,	3 ,,	2 larvae living.
No. 8	,, 30 ,,	3 ,,	all the larvae dead.
No. 9	,, 25 ,,	none ,,	
No. 10	,, 25 ,,	2 ,,	all the larvae dead.
No. 11	,, 40 ,,	2 ,,	,, ,, ,,
No. 12	,, 20 ,,	1 ,,	larva dead.
No. 13	,, 35 ,,	5 ,,	1 larva living.
No. 14	,, 30 ,,	4 ,,	2 larvae living.

Ten tubes were not used.

The cultures or extracts used were as follows:

Autolyzed extract of brewers' yeast, *B. coli*, *Staphylococcus aureus*, sterilized by autoclaving. *B. coli* and *S. aureus*, sterilized by steaming. *B. coli*, killed by chloroform and a sterile filtrate of *B. coli*. This last was several weeks old at the time of the experiment.

All the media had been kept for 48 hours and sub-cultured to test for sterility before use; ·5 c.c. = 6 % was added to each tube except in the case of the autolyzed yeast in which test ·2 c.c. = 2·5 % was added to one tube and ·5 c.c. = 6 % to the other.

Autoclaved:

	No. of Tube		
Autolyzed yeast extract...	No. 1,	2·5 % added	Hatching commenced within 5 minutes.
	No. 2,	6 % ,,	
B. coli	No. 3,	6 % ,,	Hatching commenced within 5 minutes.
	No. 4,	6 % ,,	
S. aureus	No. 5,	6 % ,,	No effect within 10 minutes.
	No. 6,	6 % ,,	

	No. of Tube		
Steam heated :			
B. coli	No. 7, 6 %	added	Hatching commenced within 5 minutes.
	No. 8, 6 %	,,	
S. aureus	No. 9, 6 %	,,	No effect within 10 minutes.
	No. 10, 6 %	,,	
Killed with chloroform :			
B. coli	No. 11, 6 %	,,	Hatching commenced within 10 minutes.
	No. 12, 6 %	,,	
Filtrate of *B. coli*	No. 13, 6 %	,,	No effect within 10 minutes.
	No. 14, 6 %	,,	

After half-an-hour.

Autoclaved cultures :	
Autolyzed yeast extract	Nos. 1 and 2, all or a high percentage hatched.
B. coli	Nos. 3 and 4, ,, ,, ,, ,,
S. aureus	Nos. 5 and 6, ,, ,, ,, ,,
Steam heated :	
B. coli	Nos. 7 and 8, all or a high percentage hatched.
S. aureus	No. 9, only one or two had hatched. No. 10, all or a high percentage had hatched.
Killed by chloroform :	
B. coli	Nos. 11 and 12, a large number but certainly not all.
Filtrate of *B. coli*	Nos. 13 and 14, no effect.

After 18 *hours.*

Autoclaved :	
Autolyzed yeast extract	Nos. 1 and 2, all hatched.
B. coli	Nos. 3 and 4 ,,
S. aureus	Nos. 5 and 6 ,,
Steam heated :	
B. coli	Nos. 7 and 8 ,,
S. aureus	Nos. 9 and 10 ,,
Killed by chloroform :	
B. coli	Nos. 11 and 12 ,,
Filtrate :	
B. coli	Nos. 13 and 14, a good proportion but by no means all hatched.

It is to be noted that considerable numbers of eggs hatched out during this period in some of the remaining 10 tubes to which no addition had been made.

After 11 *days.*

Notes regarding the progress of the larvae.

Autoclaved :	
Autolyzed yeast extract	No. 1, infected bacterial growth; larvae in second and third instar, 2 in fourth. No. 2, sterile, larvae still in first instar; no progress.

	No. of Tube
B. coli	No. 3, infected, larvae still in second instar; growing.
	No. 4, sterile, larvae still in first instar; no progress.
S. aureus	No. 5, apparently sterile, larvae still in first instar; no progress.
	No. 6, apparently sterile, larvae still in first instar; but growing.
Steam heated:	
B. coli	No. 7, ? infected } Larvae growing in first and second instars.
	No. 8, ? infected }
S. aureus	No. 9, infected, larvae mostly in second instar, growing.
	No. 10, sterile, larvae still in first instar; no progress.
Killed by chloroform:	
B. coli	No. 11, infected, and a mould growing, larvae of varied growth, first to third instars.
	No. 12, sterile, larvae in first instar; no progress.
Filtrate:	
B. coli	No. 13, infected, all eggs hatched, larvae in second or third instars; growing.
	No. 14, sterile, a number still dormant; larvae in first instar; no progress.

Unfortunately time did not permit of sub-culture tests to clear up doubtful points as to infection of tubes.

The suggestion conveyed by the number of breaksdown in sterility is that the eggs were imperfectly sterilized.

After 35 *days.*

Nine spare tubes of distilled water: many eggs had hatched in all these tubes. In three of them some colourless, fuzzy looking growth has occurred, probably of fungoid origin. In six there are no signs of any growth; in a tube that was infected with *B. coli* a few larvae had reached their third instar.

Autolyzed yeast extract...	No. 1, not much further progress; some had died.
	No. 2, 2 or 3 had reached the second instar, the rest were dead.
B. coli	No. 3, larvae had reached the second or third instar; many were dead and moulds were growing, in many cases from the mouths of the dead larvae.
	No. 4, larvae in second or third instars seemingly quite healthy; this tube is now infected.
S. aureus	No. 5, only 2 living larvae, 1 in first and 1 in fourth instar; the latter was full grown; a surface mould was growing—probable bacterial infection also.
	No. 6, only 1 third instar larva was living; a mould had almost covered the surface of the tube.

	No. of Tube
B. coli	No. 7, 4 larvae were living—all in third instar (probably infected).
	No. 8, 3 larvae were living—all in third instar (certainly infected).
S. aureus	No. 9, 2 living larvae in third instar (certainly infected).
	No. 10, all dead in second instar (apparently still sterile).
B. coli	No. 11, all dead; at least 2 larvae gained their fourth instar.
	No. 12, all dead in second instar (apparently still sterile).
B. coli	No. 13, 4 larvae living in third instar; all the others died in this stage (probably infected).
Filtrate	No. 14, a number of living larvae all still in first instar (apparently still sterile).

The history of the larvae was not continued beyond this point.

As the effect of the addition of dead cultures of bacteria to the tubes in this experiment was so much at variance with previous trials and it seemed probable that the difference was due to some variation in the eggs, an endeavour was made to check these results in the following way:

A small quantity of autolyzed yeast and dead *B. coli* culture used in this experiment were therefore added to some tubes of distilled water taken from Experiment XXIII in which eggs had been lying dormant for 100 days. These tubes were numbered 4, 5 and 6. The fluids added were autolyzed yeast and *B. coli* which had been autoclaved, and a living culture of *B. coli* as control.

To tube No. 4, about 3 % of living *B. coli* culture.
,, No. 5 ,, 3 % of sterile autolyzed yeast (autoclaved).
,, No. 6 ,, 3 % of sterile *B. coli* culture (autoclaved).

Result:

No. 4, 1 egg hatched within 2 minutes; 2 more within 5 minutes; 2 more within 10 minutes.
No. 5, 1 egg hatched within 15—20 minutes.
No. 6, 1 egg hatched within 15—20 minutes.

After an hour and a half.

No. 4, 8 out of 10 eggs had hatched.
No. 5, 1 ,, 9 ,, ,,
No. 6, 1 ,, 11 ,, ,,

These figures include the eggs hatched within the first 20 minutes.

A small quantity of living yeast cells from a wort agar culture was then transferred by platinum loop to tube No. 5, the remaining eggs hatched within 15 minutes; all of 8.

About 3 % of a living culture of *B. coli*, in broth, was added to tube No. 6; 1 egg hatched within 3 minutes; another followed within 5 minutes and 4 more within 15 minutes = 6 out of 10.

The failure of 4 eggs to hatch was probably due to their being dead.

EXPERIMENT XXV.

Yeast and yeast extracts.

Eggs sterilized by a slight variant of Method *c* were transferred by pipette into tubes containing 6 c.c. sterile distilled water.

The number of eggs in each tube varied between about 25—50.

On the 16th day an examination of tubes showed:

No. 1, 3 eggs hatched. Larvae dead.
No. 2, 3 ,, ,,
No. 3, 5 ,, ,,
*No. 4, none hatched.
No. 5, 6 eggs hatched. Larvae dead.
No. 6, 2 ,, 1 larva dead and 1 living.
No. 7, 3 ,, 1 ,, ,, 2 ,,
*No. 8, none hatched.
No. 9, 1 egg hatched. Larva dead.
No. 10, 6 eggs hatched. 5 larvae dead and 1 living.
No. 11, 10 ,, 9 ,, ,, 1 ,,
No. 12, 10 ,, Larvae dead.

* The number of eggs in these tubes was smaller than in the others, but more than 10

To tubes Nos. 1, 8, 3 and 7 ·5 c.c. (= 1 in 12) of a sterile watery extract of yeast was added.

To tubes Nos. 2, 9, 5 and 10 ·5 c.c. (= 1 in 12) of sterile autolyzed yeast extract was added.

To tubes Nos. 4, 6, 11, 12 a small mass of living yeast cells from a wort agar culture was added.

After 10 *minutes.*

Living yeasts (*S. cerevisiae*):

Numbers of eggs had hatched in tubes 6—12 and 11, but only one in tube No. 4, but others followed almost immediately.

Watery extract of brewers' yeast:

2 hatched in No. 3, a number in No. 1, none in No. 8, and only 1 in No. 7.

Autolyzed extract of brewers' yeast:

Numbers hatched in tubes Nos. 2, 9, 5, and 10.

After 45 *minutes.*

Living *S. cerevisiae*:

Numbers, probably most, of the eggs had hatched in Nos. 6, 12, and 11; a few in No. 4. The numbers were too large for accurate counting, but one got the impression that a smaller proportion of eggs have hatched in No. 4 than in the other tubes.

Autolyzed extract of brewers' yeast:

Hatching was general in tubes Nos. 2, 9, 10 and 5.

Watery extract of brewers' yeast:

None had hatched in No. 8, only 2 in No. 3; a good proportion, probably one-third, had hatched in No. 1; only 3 in No. 7 out of a large number (probably quite 50 eggs).

Tube No. 10 was infected from No. 4 to test the effect on larval growth.

After 18 *hours.*

The hatching is now general in all the tubes to which living yeasts or autolyzed yeast extract was added.

Living yeasts (*S. cerevisiae*):

The larvae in tubes Nos. 4, 6, 11 and 12 were distinctly in advance of those in tubes Nos. 2, 5, 9 and 10, to which autolyzed yeast extract was added.

Tube No. 10, inoculated from No. 4, showed no advantage to larval growth as yet.

Watery extract of brewers' yeast:

Tubes Nos. 1, 3, 7 and 8 do not show consistent results. In No. 1, 8 eggs had hatched—a fair proportion of the eggs present (about 22 to 25). In No. 3, only about 3 eggs have hatched out of some 20—25. In No. 7, 10 have hatched (about half the eggs). In No. 8, only 3 eggs have hatched out of 15—20.

After 36 *hours.*

Watery extract of brewers' yeast:

Sterility had broken down in No. 7 and hatching was general. In No. 1 no more eggs had hatched. In No. 3 hatching was general, yet there is no evidence of infection. In No. 8 only 3 eggs had hatched.

Living yeasts (*S. cerevisiae*):

In these tubes the growth of the larvae is greatest in those which contain the fewest larvae.

Autolyzed extract of brewers' yeast:

The larvae in tube No. 10 (infected) show no advantage as yet over those in Nos. 2, 5 and 9.

After 4 *days.*

Watery extract of brewers' yeast:

Tube No. 1, tests show that this tube is still sterile; many eggs remain dormant.
,, No. 7 (infected), larval progress has been rapid.
,, No. 8, sterility broke down shortly after the previous note; all the eggs have now hatched.
,, No. 3, hatching had been very general in this tube, but it still remains sterile.

Autolyzed extract of brewers' yeast:

Tube No. 9, a bacterial growth was in progress and larval development was rapid.
,, No. 2, had been infected; the larvae were progressing.
,, No. 5, remained sterile; larvae showed scarcely any progress.
,, No. 10 (infected), larvae showed varied progress; a few were well grown in third instar, others were still small in first or second instar.

After 18 *days.*

Watery extract of brewers' yeast:

Tube No. 1 (sterile), none of the larvae had passed their first moult.
,, No. 3 (sterile), none of the larvae had passed their first moult; all dead but three.
,, No. 8 (infected), varied growth in second, third and fourth instar.

Tube No. 7 (infected), larval growth had slowed down; none had yet reached their fourth instar.

Autolyzed extract of brewers' yeast:

Tube No. 5 (sterile), larvae were all still in first instar.

,, No. 2 (infected), larvae in various stages of growth in second and third instar.

,, No. 9, all killed by vigour of bacterial growth.

,, No. 10 (infected), in third and fourth instar.

Living yeasts (*S. cerevisiae*):

The larvae in these tubes had been given more yeasts. In the tubes where they were most numerous they were in third and fourth instar. In tubes Nos. 11 and 12 where fewer larvae were present a few adults had already been bred and the remainder were either full grown larvae or pupae.

After 42 days.

Watery extract of brewers' yeast:

Tube No. 1 (sterile), all were dead; none past second instar.

,, No. 3 (sterile), 3 or 4 living in second instar, the rest dead in first instar.

,, No. 8 (infected), nearly all dead in various stages.

,, No. 7 (infected), all dead but one in its third instar.

Autolyzed extract of brewers' yeast:

Tube No. 5 (sterile), a few had reached their second instar.

,, No. 2 (infected), but little, if any progress since 18th day.

,, No. 10 (infected), 1 adult reared, 1 pupa, many full-fed larvae.

Living yeasts (*S. cerevisiae*). No food since 18th day:

Tube No. 4, all dead, but 1 in third instar.

,, No. 6, all dead, but 1 in third instar.

,, No. 11, 2 adults reared; remainder dead in various stages.

,, No. 12, all dead, but 1 in fourth instar.

The history of the larvae was not continued beyond this point.

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[1] Both quotation and record are taken from Howard, Dyar and Knab (1912), Vol. I. p. 281. We have been unable to refer to this book in London. There is no reference to it in the *Zoological Record*.

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N° 2

THE USE OF INSECTICIDES AGAINST LICE.

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The scheme followed in the undernoted trials was to arrange the conditions on as nearly natural a basis for the insects as was consistent with their control and strictly comparative results, particular care being taken that the insects should have the opportunity of feeding during the experiments. Most of the remedies were those already in common use. The tests with cytisine were made at the suggestion of Dr. H. H. Dale, who very kindly supplied me with the preparation.

Cytisine is an alkaloid showing similar physiological properties to those of nicotine, but much less volatile. It was for this reason and the fact that, as it is extracted from the seeds of gorse and laburnum, an abundant supply of the raw material necessary for its manufacture is easily obtainable, that Dr. Dale suggested its trial as a remedy. (Dale and Laidlaw on the physiological action of cytisine, *Journ. Pharm. Exper. Ther.*, 1912, 3, 205.)

Preliminary trials carried out with naphthalene showed that the diffusion of vapours over the body surface beneath clothing is restricted to a very small area round the centre of distribution, in so far as it had any effect on lice. Presumably this depends partly on the curved surface of the body and partly on the difference in temperature between it and the surrounding air.

Twenty-five grams of flake naphthalene were placed in a long tube of fine muslin (nainsook) which was worn as a belt next the skin. Lice in small bags of silk gauze (motor veil chiffon), kept from collapsing by a wire gauze circle (Fig. 1), were suspended between the skin and clothing at various parts of the body above and below this belt—back, chest, abdomen, and hips—the greatest distance being 5 or 6 in. from the belt. One bag slipped from its fastenings and fell to within an inch of the belt. After a three hours' trial, it was found that the insects in the fallen bag alone were affected, some of the lice in it being paralysed, though none were actually dead.

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In a second trial 5 grams of naphthalene were wrapped up in a small packet of motor veil, and this was put into one of the bags containing a number of lice and placed next the skin. A second bag, also stocked with lice, was placed in close proximity to the first, the edges of the two bags being separated by a space of only half an inch. In the first bag, in which the lice might actually come in contact with the naphthalene, the insects were killed within 3 hours, but in the second bag, although they were not noticed to feed after the first 2 hours, they were found on examination to be still active after 6 hours; many had relaxed their hold after 11 hours, but some were still active. This bag was removed after 11 hours from its proximity to the one containing the naphthalene. When examined next morning most of the lice had recovered; only two larvae and one adult were dead out of thirty in all stages of development.

This experiment was repeated—using three bags, one on either side of that containing the naphthalene. The quantity used was again 5 grams, and the surface area of the packet was 4 square inches. After 3 hours the great majority of the lice in the central bag were dead, but three or four larvae and nymphs about to undergo a moult were active. No effect at all had been produced in the adjoining bags. After 9 hours all were dead in the central bag, but in the bags on either side a few adults only had been affected and none were actually dead, though some were partially paralysed.

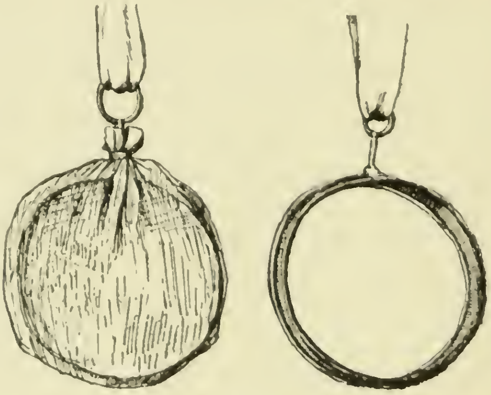

Fig. 1.—Small bags of silk gauze (motor veil chiffon) kept from collapsing by a wire gauze circle.

In order to test the relative effects of contact and diffusion of a number of remedies with greater accuracy a small apparatus was designed (Fig. 2), consisting of five wire gauze frames, each capable of being covered by a small gauze bag; they constituted a series of five small cages, each 1 in. square by ⅜ in. deep; they were fixed to a flexible bar with their edges in contact and so arranged that the whole could be tied firmly against the body, the gauze on one side coming in contact with the skin so that the captive lice could feed.

In the central chamber, referred to as "contact" in the experiments, was placed a 1 in. square of thick lint impregnated with the fluid or substance it was desired to test. This lint was carefully weighed before and after impregnation, so that the weight of substance in relation to area of surface was accurately determined. Lice were placed in all five chambers—their relative position being referred to as 2 left, 1 left, contact, 1 right, and 2 right, respectively.

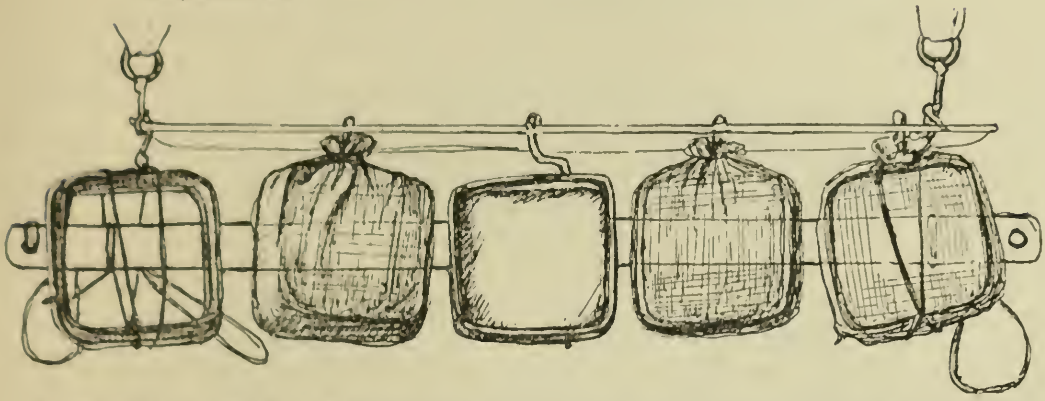

FIG. 2.—Set of five wire gauze square frames, three of them covered by small gauze bags, one with cover removed, and one in the middle containing a square inch of thick lint.

In one case three substances were tested at once, the substances being placed as follows:

REMEDY	CONTROL	REMEDY	CONTROL	REMEDY

The lice in the control chambers served to indicate either the action by diffusion, if any, or the fact that the substances tested were in no way interfering with each other's action on the lice.

The tests conducted with this apparatus were usually of 8 to 12 hours' duration, it being impracticable to sleep with it bound to the body. When the apparatus was removed at night the chambers were placed in separate boxes for a complete examination next day.

Naphthalene.

The 1 in. square of lint was dipped in a 30 per cent. solution of naphthalene in "benzole"; after the "benzole" had completely evaporated the lint held 0.133 of a gram of naphthalene; 45 lice put into the "contact" compartment were all killed within three hours. A few were immobile in chambers 1 right and 1 left, but most of them were active, as well as all those in 2 right and 2 left.

After 10 hours:

1 right ...	... 24 immobile, 3 active.
1 left ...	... 35 ,, 4 ,,
2 right and 2 left	... Many immobile, a few active.

An examination next day showed that many had recovered.

Result :

"Contact"	All killed (in 3 hours).
1 right	8 killed, 19 living.
1 left	4 ,, 35 ,,
2 right	1 ,, 24 ,,
2 left	All living.

The lint was weighed next day; the loss of naphthalene was approximately half, 0.070 gram. It was left on a shelf exposed to the air at about 65° F. On the second day crystals were still visible in the fabric with the aid of a lens. On the third none were visible, but it smelt strongly. On the fourth day it smelt faintly. On the sixth no smell was perceptible.

Sulphur.

Flowers of sulphur were dissolved in carbon bisulphide; the solution contained 15 per cent. of sulphur. The lint, after a thorough evaporation, retained 0.138 gram of sulphur.

No effect was observed on any of the lice in any of the chambers after 12 hours. Examination next morning showed the following result:

"Contact"	15 dead, 20 living.
1 right	2 ,, 14 ,,
1 left	9 ,, 12 ,,
2 right	All 28 ,,
2 left	All 20 ,,

A loss of weight (presumably by the dusting out in fine powder) to the extent of 0.034 gram took place.

The survivors were kept in a box and fed; 2 more died within the next 24 hours.

"*Cresylic Acid.*"

A few small drops were distributed over the surface of a piece of lint; by weight the quantity was 0.240 gram. After 3 hours all were immobile in the "contact" chamber, but activity was still seen in the adjoining ones. After 9 hours no movements were observed in 1 right or 1 left, but activity was still observable in 2 right and 2 left.

The cresylic acid spread on to the gauze caused a burn on the skin, and no doubt had spread also on to the gauze of the adjoining chambers, 1 right and 1 left.

Result :

"Contact"	All dead (24).
1 right	,, (27).
1 left	,, (27).
2 right	5 dead, 1 paralysed, 16 living.
2 left	3 ,, 2 ,, 21 ,,

Two of the survivors died, and one became paralysed within the next 24 hours. The lint retained its smell for 6 days. No smell was noticeable after 8 days.

Iodoform.

One gram was dissolved in 10 c.cm. of ether, and the lint was immersed in the solution. After the evaporation of the ether the lint retained 0.112 of a gram of iodoform. After 4 hours the lice in "contact" chamber were immobile, but had not loosed their hold; they were active in the other chambers. After 9 hours some of the lice in 1 right and 1 left were immobile.

Result :

"Contact"	16 dead; 3 nymphs show feeble movement.
1 right	2 ,, 15 living.
1 left	2 ,, 20 ,,
2 right	3 ,, 19 ,,
2 left	1 ,, 14 ,,

All the living were very active.

The lint retained its smell of iodoform for several weeks.

Vermijelli.[*]

0.350 gram was smeared on to the lint. After three hours the lice were active in all chambers. After nine hours none were observed to be affected.

Result :

"Contact"	3 dead, 1 feeble,	18 active.
1 right	...	20 ,,
1 left	2 dead	19 ,,
2 right	...	25 ,,
2 left	1 dead	21 ,,

Five of the lice in the contact chamber died two days after the experiment.[†]

Vaseline : Naphthalene : Kerosene Oil : Benzole.

Vaseline, 1 gram 10 per cent. solution of naphthalene in kerosene 1 c.cm. ; 30 per cent. solution of naphthalene in benzole 1 c.cm. The benzole would certainly have evaporated prior to use; 0.400 gram of this mixture was rubbed into the lint. After 3 hours : "Contact" all immobile. Some active in all the adjoining chambers. After 9 hours : Those in "contact" certainly dead ; some still active in side chambers.

Result :

"Contact"	All dead.	
1 right	2 dead,	20 living.
1 left...	2 ,,	18 ,,
2 right	...	20 ,,
2 left...	1 dead,	19 ,,

Vermijelli with Cresylic Acid.

The cresylic acid was added to a concentration of 10 per cent. ; 0.212 gram rubbed into the lint. After 3 hours : All in the "contact" were immobile ; some active in side chambers. After 9 hours : No change observed.

Result :

"Contact"	All dead.	
1 right	1 dead,	17 living.
1 left...	1 ,,	19 ,,
2 right	...	23 ,,
2 left...	1 dead,	23 ,,

Cytisine.

0.100 gram dissolved in chloroform. The chloroform was entirely evaporated before the test. After 3 hours : "Contact" all immobile ; movement occurring in all the other chambers. After 8 hours : All have relaxed their hold in contact ; some are immobile in 1 right and 1 left.

Result :

"Contact"	All dead.	
1 right	3 dead,	20 living.
1 left	2 ,,	18 ,,
2 right	...	20 ,,
2 left	1 dead,	20 ,,

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[*] An insecticide prepared by Messrs. Bowley and Son, Battersea, on the basis of Professor Maxwell Lefroy's formula, as stated in the appendix to his pamphlet, *Measures for Avoidance and Extermination of Flies, Mosquitos, Lice, and other Vermin :*

"Bolton" oil or grease (cracking point above 600°)	20 grams
(Texas) fuel oil (sp. gr. 0.86, boiling point 200° C. to 350° C.)	50 grams (58 c.cm.)
Pure soft soap	30 ,,
Water (about)	6 c.cm.

[†] It is doubtful to what extent the deaths were due to the vermijelli. No doubt some were, but in this and all the experiments an allowance must be made for natural deaths ; when using lice from a stock box some will be old and may die naturally during the course of the experiment.

Vermijelli with Phenol.

The phenol was added to a concentration of 10 per cent. and 0.219 gram rubbed into the lint. After 3 hours: Contact, all immobile; movement observed in all the other chambers. After 9 hours: In contact all have relaxed their hold; movement still to be seen in the other chambers.

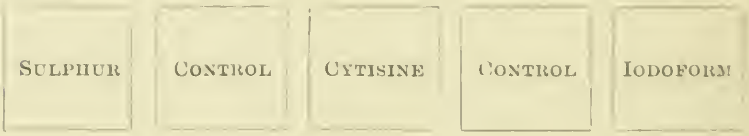
Result:

"Contact"	All dead.
1 right	1 dead, 21 living.
1 left	21 ,,
2 right	2 dead, 17 ,,
2 left	1 ,, 22 ,,

Iodoform, Cytisine, and Sulphur.

A further trial was carried out between iodoform, cytisine, and sulphur, all of which, being very slow in dispersion, would presumably be effective for a long period.

The pieces of lint used were those employed in the previous tests; having been freely exposed to the air in a room since the previous trials, that with sulphur for one month, with iodoform for twenty-three days, and with cytisine for seven days. The trials were all made at one time. The chambers 1 right and 1 left were stocked with lice to act as controls in case of diffusing effects. The position in the chambers was as follows:



After 3 hours—Sulphur: lice clustered together, but apparently all living. Cytisine: nearly all immobile. Iodoform: a few immobile—one or two possibly dead, others active. After 9 hours—No change to record.

Result:

None of the controls in intermediate chambers were affected.

Sulphur	1 dead, 19 active.
Cytisine	2 dead, 17 paralysed, 1 active.
Iodoform	10 dead, 3 paralysed, 5 partially paralysed, 3 active.

All the living insects were kept two days and given opportunity of feeding.

The final result then was:

Sulphur	19 living;	mortality 5 per cent.
Cytisine	2 ,,	,, 90 ,,
Iodoform	2 ,,	,, 90 ,,

The action of both iodoform and cytisine after so considerable a lapse of time was hopeful and rendered further tests desirable.

To impregnate the pieces of lint the cytisine was dissolved in chloroform and the iodoform in ether. The quantities used were 6 mg. of cytisine and 5 mg. of iodoform on pieces of lint 1 in. square.

After 5 hours—Cytisine: all but one seem paralysed. Iodoform: 6 appear paralysed and 4 of these may be dead; 22 can walk, and one pair are copulating. The trial was then stopped and the insects boxed for examination next day.

Result :

Cytisine ...	6 are dead, the rest partially or wholly recovered.
Iodoform ...	1 is dead, the rest apparently recovered.

Two days after the trial :

Cytisine ...	...	2 more have died—	8 out of 28.
Iodoform ...	...	7 ,, ,,	8 ,, 28.

The net results of the trials is to suggest that the diffusive effects of all the substances, except possibly naphthalene, is so slight as to reduce them all to a common level of contact remedies. Viewed in this light it is necessary to impregnate or thoroughly dust underclothing with them to obtain any effect. For this purpose the cost of iodoform and cytisine is likely to prove prohibitive, although their use might be practical for impregnating the seams of trousers in the neighbourhood of the fork. For this purpose the long period of their effectiveness would be a set-off against the high first cost of treatment. Cytisine has the advantage of being odourless and quicker in action than iodoform.

Naphthalene used as a powder is likely to be wasted, and when made up into sachets with sawdust, as suggested by Noel,[1] or moulded into tablets, will probably lose its effectiveness just in proportion to the slowing of its evaporation. If used in a form in which garments can be impregnated with it, it is an almost perfect remedy, as it kills quickly, and where there is any risk of infection is probably the best means available. The chief objection is its rapid evaporation ; by the second day after impregnation of flannel shirting its power has either greatly waned or has already departed, according to the original quantity used. As Kinloch[2] has already pointed out, nothing is gained by the addition of iodoform to naphthalene as in the N.C.I. powder.

Insecticidal Effect when Substances are Used in Diluted Condition to Impregnate Cloth.

Attention was next directed to remedies of low cost and slow action with a view to testing their effectiveness in preventing breeding when used to impregnate garments. For this purpose the system and apparatus used in the previous experiments were not suitable, because it was necessary to keep adult lice confined for several days in order that there might be ample time for egg laying and for the eggs to develop. The following method was found to work satisfactorily in preliminary trials.

Shallow card boxes, 1 in. in diameter by ⅜ in. deep, were lined at bottom and sides with flannel shirting material that had been washed and dried by ordinary laundry process and then dipped in the solution it was desired to test, and dried off rapidly. The open top of the boxes was covered with fine chiffon, through which the insects fed. These boxes were kept by day in a linen bag suspended beneath the clothing ; at night they were placed beneath a belt with the chiffon against the skin, the period available for feeding being about seven hours.

Three, or more usually four, females and two males were placed in each box; they were removed from the boxes after a week; the eggs laid were then counted and their position noted. All those laid *elsewhere than on the treated material* were removed and the test continued until the eggs hatched and the young lice had passed the first moult and recommenced to feed.

Control (untreated flannel used): 1 died; 121 eggs were laid on the flannel, none elsewhere. *Result:* The eggs hatched in due course, the larvae fed, moulted, and recommenced to feed.

Sulphur.—Flannel dipped in colloidal solution of sulphur: 1 female died; 117 eggs laid on flannel, none elsewhere. *Result:* Same as control.

Vermijelli.—Flannel treated with a 4 per cent. solution of vermijelli in water: 2 females died, 72 eggs on flannel, 1 on box, 6 on gauze cover. *Result:* Same as control.

Vermijelli.—Flannel treated with a 4 per cent. watery solution of vermijelli, to which cresylic acid had been previously added to a concentration of 5 per cent.: 1 male died, 74 eggs laid on the flannel, and 14 on the gauze cover. *Result:* Same as control.

Vermijelli and Cresylic Acid.—Flannel treated with a 10 per cent. watery solution of vermijelli, to which cresylic acid had been previously added to a concentration of 5 per cent.: 2 females and 1 male died, 70 eggs laid on flannel, and 21 on the gauze cover. *Result:* Two or three larvae died, otherwise same as control.

Vermijelli and Naphthalene.—Flannel treated with a 5 per cent. watery solution of vermijelli, to which 20 per cent. of naphthalene had been added before making the solution. *Result:* All the lice killed within 1 hour, no eggs laid.

In these experiments the tests were commenced as soon as the prepared flannel was thoroughly dry.

The last remedy is open to the objection already recorded against naphthalene, namely, the short period—about two days at most—for which it remains effective. The use of naphthalene in this series, employing the box method, however, affords a standard of comparison with the gauze bag trials of the earlier series, and indicates that the condition of these later trials favours the remedies as against the insects. In making this comparison it must be borne in mind that the quantities of vermijelli used in the later series was very much smaller than in the former when the undiluted preparation was smeared over the lint.

In the further trials with the box method, the flannel after treatment and drying was generally exposed to the air in a room with a temperature of about 65° F. for a longer or shorter period before the trial.

The following additional preparations were experimented with: An emulsion consisting of equal parts of soft soap and "crude liquid carbolic acid"*; also several preparations of a rosin soap—crude carbolic acid emulsion allied to the Isthmian mosquito larvicide used by the Panama Canal authorities. This preparation did not give a perfect emulsion and consequently proved unreliable for impregnating flannel. The same remarks apply to emulsions of soft soap and crude carbolic, in which less than 40 per cent. of soft soap was used.

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*The suppliers state that the fluid supplied consists chiefly of cresols mixed with a little phenol, and a certain proportion of higher homologues.

Flannel Treated Two Days before Use; Period of Test 18 Hours.

Five per cent. solution of an emulsion consisting of equal parts of soft soap and "crude carbolic." Result: 4 females and 1 male killed; 1 male paralysed, no eggs laid.

Five per cent. solution of vermijelli to which 20 per cent. of naphthalene had been added. Result: None of the adults were killed and a number of eggs were laid.

The contrast between the results of the first and second trials of the vermijelli-naphthalene preparation affords clear evidence of the rapid evaporation and consequent deterioration of effectiveness of this remedy if a couple of days be allowed to elapse between the impregnation of the flannel and its use. The smell of naphthalene was only faintly discernible when the test was made.

A method was next instituted to test not only the effectiveness of the remedy as against lice, but its action on the skin.

Pieces of flannel, about 2 in. by 3 in. were treated and dried. A pocket of fine chiffon was then stitched on to the flannel, a number of lice inserted, and the open end stitched down, thus imprisoning the insects between the gauze and flannel. These pieces of flannel were then pinned on to the inside of the shirt in a suitable situation for the insects to feed through the chiffon, which was set against the skin.

Series I.—The following trials were made with flannel treated two days previously to test with:

1. A 3 per cent. solution of an emulsion consisting of 100 parts crude carbolic, 20 parts naphthalene, 70 parts of soft soap.
2. A 3 per cent. solution of an emulsion consisting of 100 parts crude carbolic and 70 parts of soft soap.
3. Treated with a solution of naphthalene in benzole at the time of trial; the flannel held 0.05 gram by weight.

After 20 hours the results were as under:

1. Two dead, 16 living; a few eggs laid.
2. Nine dead (this number includes all the females). 11 living (all males or nymphs); no eggs laid.
3. All living (20); a number of eggs laid.

No irritation caused to skin.

Series II.—In another series the flannel was treated one day prior to use, the remedies being:

4. A 10 per cent. solution of a kerosene soft soap emulsion containing naphthalene.
5. A 5 per cent. solution of an emulsion consisting of 100 parts soft soap, 100 parts crude carbolic, and 20 parts naphthalene.
6. A 5 per cent. solution of an emulsion consisting of 100 parts soft soap with 100 parts crude carbolic acid.

After 24 hours' trial the results were as under:

4. Seventeen living, 4 dead; a few eggs laid.
5. Fifteen living, 5 dead, 1 paralysed; 1 egg laid.
6. One living, 19 dead; no eggs laid.

No irritation was caused to the skin.

A Jaeger flannel shirt was then impregnated with a 10 per cent. solution of an emulsion consisting of equal parts of soft soap and crude carbolic. The shirt took up 1,000 c.cm., and after wringing out held 600 c.cm. After drying, lice were imprisoned in pockets of chiffon stitched on to the inner side of the shirt in the following positions: (1) On the chest; (2) in the lumbar region of the back; (3) on the outer side of the upper arm.

None were killed within 3 hours. After 5 hours a number in situations 1 and 2 were either dead or paralysed. After 10 hours all or nearly all were dead at 1 and 2, but many were still active on the arm. After 18 hours all were dead in situations 1 and 2, while of those at 3, 7 were dead and 4 paralysed. No eggs were laid. The effect on the skin was a slightly disagreeable irritation at the armpits and lumbar region. The lice fed at the commencement of the trial.

The test was repeated, using a 5 per cent. solution of the same emulsion; the wringing out process was rather more drastic—the garment retaining only 500 c.cm. It was dried during the night, lice being confined under covers of chiffon on the following day in the same situations as previously. After 10 hours some were still living in positions 1 and 2, and all were active at 3.

First Day.—After 24 hours all were dead in 1 and 2, but a few still lived in 3. No eggs were laid. Pockets 1 and 2 restocked.

Second Day.—Twenty-four hours later all were dead in the restocked pockets, also the survivors in 3. No eggs were laid.

Third Day.—The shirt was worn the next 48 hours without restocking.

Fourth Day.—The old pockets were then restocked.

Fifth Day.—Twenty-four hours later a number had died in pockets 1 and 2, but others were still living; in 3 a few were dead, others active. The frayed condition of the old pockets 1 and 2 necessitated their renewal; they were renewed and restocked. No. 3 was left.

Sixth Day.—After another 24 hours it was found that many of the lice were still living in 1 and 2; those in 3 were now all dead—a few eggs having been laid. No. 3 was not restocked. Pockets 1 and 2 were left, and a new one (No. 4) was placed on the upper part of chest and stocked.

Seventh Day.—After a further 24 hours nearly all the survivors in No. 1 were dead. No eggs laid; in No. 2 some were still active. In No. 4 all were active.

Eighth Day.—In No. 1: 9 were dead, 4 living; 36 eggs had been laid. In No. 4: 4 were dead, 16 living; 40 eggs had been laid.

The garment still smelt of carbolic, perhaps sufficiently to have some deterrent effect, but the lethal effect had evidently passed away.

No unpleasant skin irritation was noticed; a slight tingling that could just be discerned was experienced on two occasions after the start.

It may be concluded that the practical effect of the impregnation lasts about six or seven days, while the smell would possibly act as a deterrent for a few days longer.

Another shirt was impregnated with a 3 per cent. solution; after wringing, it was found to hold 500 c.cm. It was dried during the night and lice were placed in a pocket on the chest next day. After forty-eight hours, although one or two were dead, the great majority were still active.

Cytisine.

Further experiments with cytisine were made with the kind assistance of my friend Dr. A. J. Ewins, to ascertain the minimum quantity that would be of practical service for impregnating garments, and the period during which this preparation retains its efficacy.

The method employed was to impregnate pieces of flannel shirting, similar to that used for army shirts, with weak solutions. After thoroughly drying, a piece of fine silk gauze was stitched on, forming a pocket, under which the lice were placed. The piece of flannel was then pinned on the inside of the shirt, so that the insects were able to feed through the silk gauze.

Using 1 mg. per square inch of surface, 7 out of 16 lice were killed in two days, but none of the survivors died within the next three days.

Using 5 mg. per square inch, 11 out of 12 were killed within the first 24 hours, one young larva remaining active. The pocket was then restocked, and 10 out of 15 were killed within two days. A further restock resulted in 6 out of 14 dying in the next two days. A number of eggs were laid by the survivors. The piece of flannel was then completely extracted with chloroform, with the result that only traces of cytisine were recovered.

Using 10 mg. per square inch:

1st stock: 19 lice were all killed within 24 hours.
2nd stock: 15 lice—9 were killed and 3 paralysed within 24 hours; 3 remained active.
3rd stock: Of 15 lice, 10 were killed and 1 paralysed within 24 hours; 3 remained active.
4th stock: Of 14 lice, 10 were killed and 4 remained active after 24 hours.
5th stock: Of 12 lice, 3 were killed and 9 remained active after 24 hours.
6th stock: Of 12 lice, 9 were killed and 3 were paralysed within 24 hours.
7th stock: Of 10 lice, 8 were killed and 3 remained active after 28 hours.
8th stock: Of 10 lice, all were active after 24 hours, but within the next 26 hours all were killed.
9th stock: Of 9 lice, all were active after 24 hours; 6 were active after 48 hours; 5 after 72 hours; 4 after 96 hours; 5 dying during the course of the test.

The apparent falling off in lethal power at the period of the 6th stock was probably due to the fact that a cool day checked sweating.

Although quite satisfactory from an experimental point of view, there are two apparently insurmountable difficulties which prevent the use of cytisine as a practical remedy. First, its possible toxic effect if used to impregnate garments. At a concentration which, after drying, would leave 10 mg. per square inch, the quantity required to treat a shirt would amount to some 12 to 15 grams; while, if the absorption over a large area of skin proved to be cumulative, the presence of such a quantity might lead to serious or even fatal poisoning. Secondly, the high cost of production would tend to make it an extravagantly expensive remedy.

These latter trials suggest that a practical remedy may be found for preventing the spread of lice among troops by the use of a "crude liquid carbolic acid" and soft soap emulsion for the impregnation of shirts and underclothing. The emulsion should consist of 45 per cent. to 50 per cent. of soft soap, combined by heating with 50 per cent. to 55 per cent. of the crude carbolic. The strength of the solution used to impregnate the garments should be 5 per cent. of the emulsion in warm water, 3 per cent. being too weak for practical use; while solutions above 5 per cent. might cause irritation to the skin. After dipping, the garments should be wrung and thoroughly dried before wearing. A normal sized flannel shirt after wringing will retain about 500 to 600 c.cm. of the solution, and the cost would probably, apart from labour, be about one halfpenny per shirt.

What is now required is a test on a practical scale to ascertain (*a*) what percentage of men are likely to have irritable skins which might be susceptible to the small percentage of cresols in the shirt, and (*b*) to ascertain the efficacy of the remedy to keep men free from lice in the field.

I desire to record my thanks to my colleague, Dr. Harriette Chick, for valuable suggestions and advice.

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No 3

THE

IMPROVEMENT OF FLY-SPRAYING FLUIDS

AND THE

CONTROL OF EXPERIMENTAL TRIALS.

In the course of a number of experimental trials of different fly-spraying fluids I found, as is, I believe, the general experience, that when these fluids are used in accordance with the directions they knock down or stupefy but do not kill all the flies; a varying percentage recover after an hour or two. This is especially noticeable when the temperature is at or above 72° F. In some cases, where the spray seemed fairly effective at room temperature (60–65°), it was only necessary to transfer the stupefied flies to a warm room (at 75° F.) when they at once began to recover and speedily regained their full activity. A number of parallel experiments proved that the use of an emulsion of kerosene oil and soft soap, diluted about 1 in 10, killed the flies within a period of twelve to eighteen hours, although it did not stupefy them speedily, as do most of the proprietary sprays. I therefore tried an admixture of the quick action sprays with the oil emulsion fluid, and found that the combination gave excellent results, quickly stupefying the flies on the one hand and preventing their recovery on the other. The oil emulsion used consisted of 3 parts of soft soap, completely melted by heat in 15 parts of water, a proportion up to 100 parts of kerosene or other light burning oil is then added very gradually with thorough agitation by stirring or shaking in a closed vessel between each addition. The resultant emulsion can be kept indefinitely, and may be diluted with water to any extent. For addition to the fly spray allowance must be made for the extent to which they are diluted for use—the quantity of oil emulsion required being 1 in 10 to the water content of the fluid.

[380 16]

The following experiments with a quick action spray with and without the oil emulsion will serve to illustrate the value of the addition.

Experiment 1.

Two wire traps containing large numbers of flies, caught within the previous two hours, were used.

Cage A.—The quick action fluid was diluted 1 in 30 (by the directions 1 in 40 should have been sufficient); this was used to spray the flies in cage A for two minutes.

Cage B.—To 20 c.cm. of this mixture 2 c.cm. of the oil emulsion was added, and the flies in cage B were sprayed with the mixture for two minutes.

The flies in both cages were all incapable of flight or active movement within fifteen minutes, the temperature being 65° F. Both cages were placed in a warm room at 75° F.

After three hours, at least half of the flies in cage A were actively flying or walking, while in cage B only five or six were active.

After seven hours: In cage A 32 were dead and 328 active (mortality, 9 per cent.). In cage B 290 were dead and 52 were active (mortality, 84 per cent.).

Experiment 2.

The flies were sprayed for two and a half minutes; almost all the flies in both cages were down within fifteen minutes. The cages were then put into a warm room at 75° F. After two hours a large number had recovered in the A cage, but only 5 in B. Both cages were left for the night in the warm room.

On the following day a count showed: Cage A, 196 dead, 193 living, mostly active (mortality, 50 per cent.). Cage B, 247 dead, 19 living, but only 5 active (mortality, 91 per cent.).

As an instance showing the susceptibility of flies to drought and the care necessary in the controlling of experimental trials the following may be quoted:

Experiment 3.

Flies (*Musca domestica*) were captured in two wire cages between 1 p.m. and 2.30 p.m. in the same room, the cages being placed about 12 in. apart. The flies in cage A were kept without food or water in a room temperature about 65° F. until 5 p.m. (two and a half hours). In cage B they were kept in the same room, but a plug of cotton-wool soaked in weak syrup was placed in the cage. At 5 p.m. this was removed, and both cages were placed in a warm room at 75° F. for seventeen hours.

In cage A 20 out of 63 were dead (mortality, 31 per cent.). In cage B 6 out of 81 were dead (mortality, 7 per cent.).

A. BACOT,

Medical Entomologist, Lister Institute.

No. 4

YELLOW FEVER COMMISSION

(WEST AFRICA)

ENTOMOLOGICAL REPORT.

REPORT

OF THE

ENTOMOLOGICAL INVESTIGATION

UNDERTAKEN FOR THE

YELLOW FEVER (WEST AFRICA) COMMISSION

FOR THE YEAR AUGUST, 1914, TO JULY, 1915

BY

A. W. BACOT, F.E.S.

(*Entomologist to the Lister Institute of Preventive Medicine.*)

An account of a research concerning the Bionomics of STEGOMYIA FASCIATA, *and the apparatus and methods employed; with some notes concerning the life history of allied species, and the effects of insecticides and salt water on the immature stages of* S. FASCIATA; *with 9 Charts, 27 Figures and 29 Plates.*

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I. INTRODUCTION.

The work with which this Report deals was carried out at Freetown, Sierra Leone, between the months of August, 1914, and August, 1915. A few experiments, for which there were no suitable facilities at Freetown, were performed at the Lister Institute of Preventive Medicine during October, 1915, with material resulting from eggs brought home from West Africa.

The original scheme of work in connection with the incidence of Yellow Fever in West Africa had to be largely modified owing to the outbreak of War. In the absence of clinical and pathological workers to co-operate with, the greater part of the energies of the entomological section were devoted to the elucidation of questions concerning the bionomics of *Stegomyia fasciata*, the main endeavour being to supplement and substantiate the already very considerable knowledge of the life history of this species, which we owe to Finlay, Goeldi, Reed and Carrol, Boyce, Newstead, Dupree and the members of the French Committee, as well as numerous other workers.

Time did not permit of the investigation of problems connected with the possibility of a seasonal variability. For instance, the tendency of eggs sometimes to hatch immediately they are placed in water, and at others to defer hatching for an indefinite period; or, again, the question of the period for which different batches of eggs remain viable, may be associated with the season of the year in which the parents producing the eggs are reared, but to clear up these and similar points would necessitate the continuance of the research over several seasons.

Charts of the temperature and humidity records in the various places where experimental work was carried out will be found in the section dealing with the length of the adult life (pages 64 and 92-101). Owing to delays in despatch and transit the thermometers were not available during the earlier months of the research. In order to give some idea of the conditions during the months of August, September, October and November, a summary of the general meteorological observations in Freetown for the year 1914 is included. The temperature of the water used for egg hatching and larvæ breeding would be, unless stated to the contrary, about 80° F.

It is with much pleasure that I avail myself of this opportunity of expressing my thanks to the members of the West African Medical Staff for their kindness in endeavouring to make my tour as pleasant and effective as possible; to Dr. W. Allan and Dr. J. S. Pearson and other friends, for placing at my disposal collections of mosquito larvæ taken in Freetown, and especially to Dr. G. G. Butler, who, in spite of the exceptional pressure of official work, brought me many collections of larvæ that he had made in the district surrounding Hill Station.

From the material collected and bred at Freetown sets of specimens of the prevalent species of mosquitoes were forwarded to the Liverpool and London Schools of Tropical Medicine, and to Mr. F. W. Edwards, of the British Museum of Natural History a small collection of specimens which it seemed possible might be of service to the National Collection. Amongst this latter material was a new species of *Eretmopodites*, of which Mr. Edwards is publishing a description in the "Bulletin of Entomological Research," Vol. VI., Part 4, page 362.

I take this opportunity of acknowledging my indebtedness to Mr. Edwards' (1912) "Keys of the African *Culicidæ* other than *Anopheles*," which I found of great assistance, and my thanks are due to their author for his kindness in checking and confirming my determinations.

My thanks and acknowledgments are also due to Messrs. Bruce F. Cummings, W. L. Distant, Stanley Hirst, G. Meade Waldo, D. J. Scourfield and H. M. Woodcock, for assistance in identifying species dealt with in this Report.

II.—INTRODUCTORY NOTES ON THE DISTRIBUTION OF MOSQUITOES IN FREETOWN (SIERRA LEONE) AND ITS VICINITY.

Mosquitoes are so far from being the omnipresent nuisance in Freetown that one is led to expect in the Tropics generally, and on the West Coast of Africa in particular, that there is a danger of being lured into a false sense of security leading to the lapse of all precautions.

Their apparent absence and actual rarity are partly due to the natural advantages conferred by the porous nature and contour of the soil, and largely to the efficiency and activity of the Sanitary Department in the inspection of compounds and the destruction of larvæ. The evidence afforded by a mosquito hunt beginning in the central area of the town and extending beyond its bounds is eloquent in its testimony of what has been accomplished.

In the outlying areas of the town and the adjacent villages much remains to be done, especially in the matter of the efficient drainage of surface water.

General observation and search for adults lead to the conclusion that *Culiciomyia nebulosa* is the dominant species within the central area, but if the larval stages be included in the search, and the larval collections made by the Sanitary Department are taken into consideration, it soon becomes obvious that an adult census is but a poor guide to the actual facts.

As regards breeding and general distribution the species of *Stegomyia*, especially *fasciata*, easily lead. Any relaxation of the Sanitary Administration in the matter of mosquito work would inevitably be accompanied by a rapid increase in the adult *Stegomyia* population.

It is even now an open question if adults of *C. nebulosa* are really more numerous than those of *S. fasciata*. While the former is comparatively casual in its selection of resting places—any damp or shaded wall in an outbuilding will do, and its breeding habits tend to produce sudden and very noticeable outbursts of adults—*S. fasciata* is exceedingly careful and able in hiding away, while it begets larvæ with a steady persistency in any available breeding place without a sign of an adult being seen.

The *Anopheles* species, *Pyretophorus costalis* and *Myzomyia funestus*, are much more restricted in distribution and breeding grounds, although the places affected by the last-named are of large extent where they do occur. Larvæ of *P. costalis*, though common and generally distributed in rock and gutter pools, are few in number compared with the swarms of *S. sugens* larvæ which are present in these situations in the outlying districts of the town (*see* Plates 8, 10 and 11, pp. 12 and 13). They also occur not infrequently within its central area, and are sometimes met with in tins and even in water-holes in trees. *M. funestus* is restricted in its breeding places and is only occasionally present in isolated rock pools, its regular *habitat* being the shallow hard-bottomed swamps which occur on the laterite flats at the foot of the hills, both to the east and west of Freetown (*see* Plates 13, 14 and 15, pp. 14 and 16). During the rains the surface water flows slowly out from the foot of the hills across these flats, covering wide areas with water trickling through the roots of the grass and forming shallow pools at frequent intervals. The pools are often tenanted by tadpoles, the mosquito larvæ being chiefly found among the roots of the grasses—the frogs or toads with which these swamps abound being no doubt responsible for the absence of mosquito larvæ in the open pools. The surface water cut between Alligator River and Congo River to the south of the railway has benefited the western end of the town by drying a considerable area of the flats and thus restricting the possible breeding grounds of *M. funestus*. On the east, however, above Fourah Bay and along the rail to Kissy large tracts of these flats are still in their natural condition.

Holes in trees, a favourite breeding situation with several species of *Stegomyia* and *Ochlerotatus*, have received special attention in the past, so that it requires careful search to find any within the central area of the town (*see* Plates 1 and 2, page 6). Small rock pools are however, only too plentiful in its outer areas, and these, together with old and irregularly graded road side drains, constitute possible breeding places, which must be a constant source of anxiety to the sanitary authorities, pending the extension of the admirably designed newer drainage system to the outer districts of the town (*see* Plates 4, 5 and 8, pp. 8, 9 and 12). Tins, jars, bottles, tubs, etc., would still seem to be the chief strongholds of *S. fasciata*; fortunately, as indicated above, the vigilance of the compound inspections prevents more than a tithe of the larvæ reaching maturity.

Plants.—The axils and central whorl of leaves of a number of plants provide breeding accommodation for a certain number of species during the rains. Of these, *Uranotænia ornatus* and a new species of *Eretmopodites** seem to be restricted to the water in these plant reservoirs. *Stegomyia simpsoni*, though most commonly reared from collections of larvæ taken from plants, certainly breeds in root holes also, while a few ubiquitous species, such as *Stegomyia fasciata* and *Culiciomyia nebulosa*, are occasional tenants.

The breeding of mosquitoes in plants opens up a difficult problem, as two of the plants, the banana and cocoa yam, are valuable sources of food supply, while another, the "Cock Hat" tree, a species of *Dracæna*, is a serviceable and largely used hedging plant. There are two species of this plant grown in Freetown and its vicinity, only one of which accumulates water in the central whorl and axils of its leaves; both species are used for hedging, but the water holder much more frequently than the other. Possibly there may be some advantage, such as speed of growth, but unless this is weighty it would be well to discourage its use (*see* Plates 16 and 17, page 17).

The feeding tendencies of the specimens reared from larvæ taken in these plants were tested in regard to their willingness to suck human blood. The abilities in this direction of *S. fasciata* or *G. nebulosa* (occasionally present) need no comment. *S. simpsoni* was found to be as insatiable as *fasciata*. *Eretmopodites* would feed lustily, but only at long and irregular intervals. *Uranotænia ornatus*, bred only from these situations, invariably refused to bite or feed at all, although offered both honey and syrup.

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* Described by Mr. F. W. Edwards in the "Bull. of Entom. Research" under the name *dracænæ*.

Plate No. 1.
Roots of a Cottonwood tree. Tower Hill.

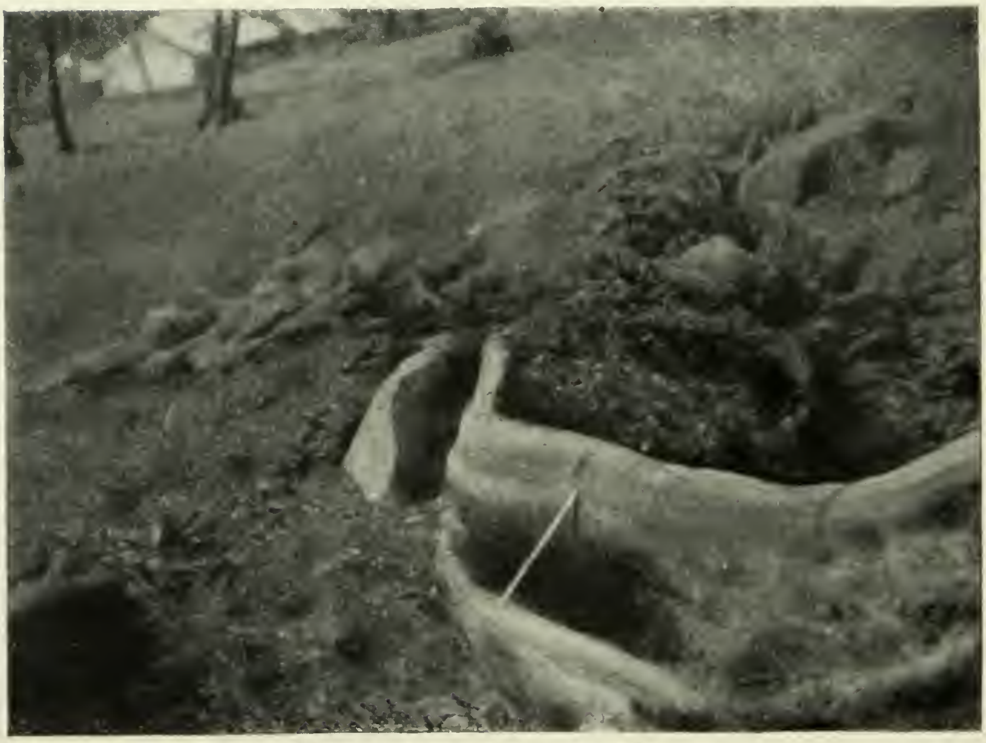

Plate No. 2.
Waterhole in the roots of the same tree. Larvæ of *Stegomyia fasciata*, *S. simpsoni*, *S. luteocephala*, *Ochlerotatus minutus*, *O. apicoannulatus*, *Culiciomyia nebulosa* and *Anopheles costalis* were taken from this pool.

LIST OF MOSQUITOES IDENTIFIED FROM VARIOUS LOCALITIES, MOSTLY BRED FROM LARVÆ.

CENTRAL AREA OF TOWN.

Tower Hill.

Larvæ from water holes in cottonwood tree (*see* Plates Nos. 1, 2 and 3, pp. 6 and 8) :—

Anopheles (*Pyretophorus*) *costalis*, Lw. ...	... two specimens.
Stegomyia fasciata, F.	... in numbers.
,, *simpsoni*, Theo.	... in numbers.
,, *luteocephala*, Newst.	... in numbers.
Ochlerotatus apicoannulatus, Edw. ...	... in numbers.
,, *minutus*, Theo.	... a few.
Culiciomyia nebulosa, Theo.	... a few.
Culex decens, Theo.	... in numbers during November.

Larvæ from rock pools (*see* Plates Nos. 4, 5 and 6, pp. 8 and 9) :—

Stegomyia sugens, Wied	... swarms.
,, *fasciata*, F.	... several.
,, *luteocephala*, Newst.	... one specimen.

Compound at Colonial Hospital.

Larvæ in old tins and broken bottles :—

Stegomyia fasciata, F.	... numbers.
Culiciomyia nebulosa, Theo.	... numbers.

Yard, 26, Westmoreland Street.

Larvæ in tins, bowls, etc. :—

Stegomyia fasciata, F.	... numbers.
Culiciomyia nebulosa, Theo.	... two or three broods.
Eretmopodites quinquevittatus, Theo. ...	... several.

Gloucester Road.

Surface water drain :—

Anopheles (*Pyretophorus*) *costalis*, Lw. ...	... one specimen.

Circular Road.

Surface water drain :—

Anopheles (*Pyretophorus*) *costalis*, Lw. ...	... one specimen.

Plate No. 3.
The root hole in Cottonwood tree filled with cement.

Plate No. 4.
Rock Pool which contained larvæ of *Stegomyia sugens.* Tower Hill.
The white streak is a foot rule.

MOSQUITO BREEDING POOLS.

Plate No. 5.
Rock Pool which contained larvæ of *Stegomyia sugens*, *S. fasciata*, *and S. luteocephala*. Tower Hill.

Plate No. 6.
The same pool filled with cement.

Collections of Larvæ received from various Houses and Compounds in the central area of the Town, including the Rest House and Nursing Home.

Stegomyia fasciata, F.	numerous.
Culex tigripes, Grp.	two or three.
Culiciomyia nebulosa, Theo.	numerous.

Campbell Street.

A tin from a compound :—

Megarhinus (*Toxorhynchites*) *brevipalpis*, Theo.	one specimen.
Ochlerotatus apicoannulatus, Edw.	several specimens.
Stegomyia frazeri, Edw.	one specimen.

Post hole near Saunders Brook.

Stegomyia sugens, Wied.	numbers.

Hill Station.

Rock pools :—

Anopheles (*Myzomyia*) *funestus*, Giles	two or three.
Stegomyia sugens, Wied.	numbers.
Ochlerotatus minutus, Theo.	swarms.
,, *simulans*, Cart.	one specimen.

Water hole in a tree :—

Stegomyia frazeri, Edw.	one specimen.
Ochlerotatus minutus, Theo.	numbers.

Lumley Road.

Bolt holes in cement foundation of sand elevator :—

Stegomyia sugens, Wied.	numbers.
Culiciomyia nebulosa, Theo.	numbers.

Wilberforce.

Old bottle, wayside ditch :—

Stegomyia fasciata, F.	numbers.

Old tins, rubbish heap :—

Ochlerotatus minutus, Theo.	numbers.
Eretmopodites quinquevittatus, Theo.	several.
,, *chrysogaster*, Grah.	a few.
,, *inornatus*, Newst.	one specimen.

Rock pools on wayside boulders (*see* Plate No. 7, page 11) :—

Anopheles (pyretophorus) costalis, Lw.	one or two.
Stegomyia sugens, Wied	numbers.
,, *luteocephalus*, Newt.	a few.

ROCK POOL NEAR WILBERFORCE STATION.

Plate No. 7.

A breeding hole on a boulder on Wilberforce Hill; larvæ of *Stegomyia sugens*, *S. luteocephala* and *Anopheles costalis* were taken here. The white streak is a foot rule measure showing the small size of the hole.

Murray Town.

Road side rock pools :—

Anopheles (pyretophorus) costalis, Lw.	several.
Stegomyia sugens, Wied.	numbers.
,, *fasciata*, F.	numbers.

Cline Town.

Small tank in a compound :—

Anopheles (pyretophorus) costalis, Lw.	several.
Culex tigripes, Grp.	one or two.
Ochlerotatus apicoannulatus, Edw.	a few.

MOSQUITO BREEDING POOL.

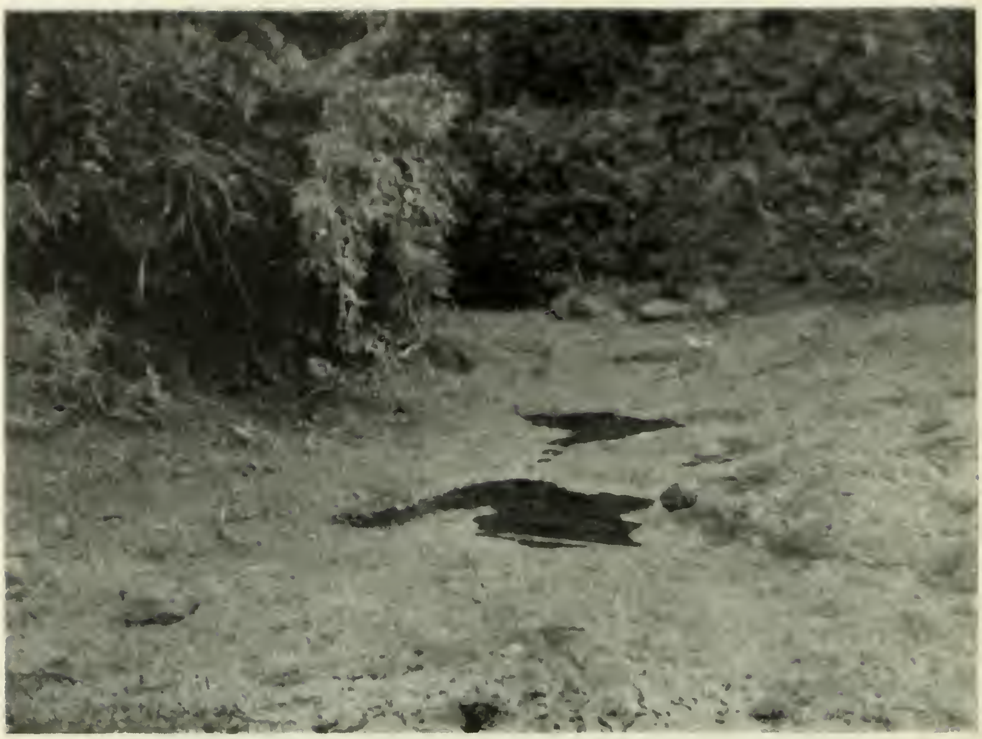

Plate No. 8.

Pools of water in bed of surface water drainage channel near Kissy Road Cemetery ; contained larvæ of *Stegomyia sugens* and *Anopheles costalis* in May. In July this channel was the bed of a torrential brook.

BREEDING GROUND OF *ANOPHELES* MOSQUITOES.

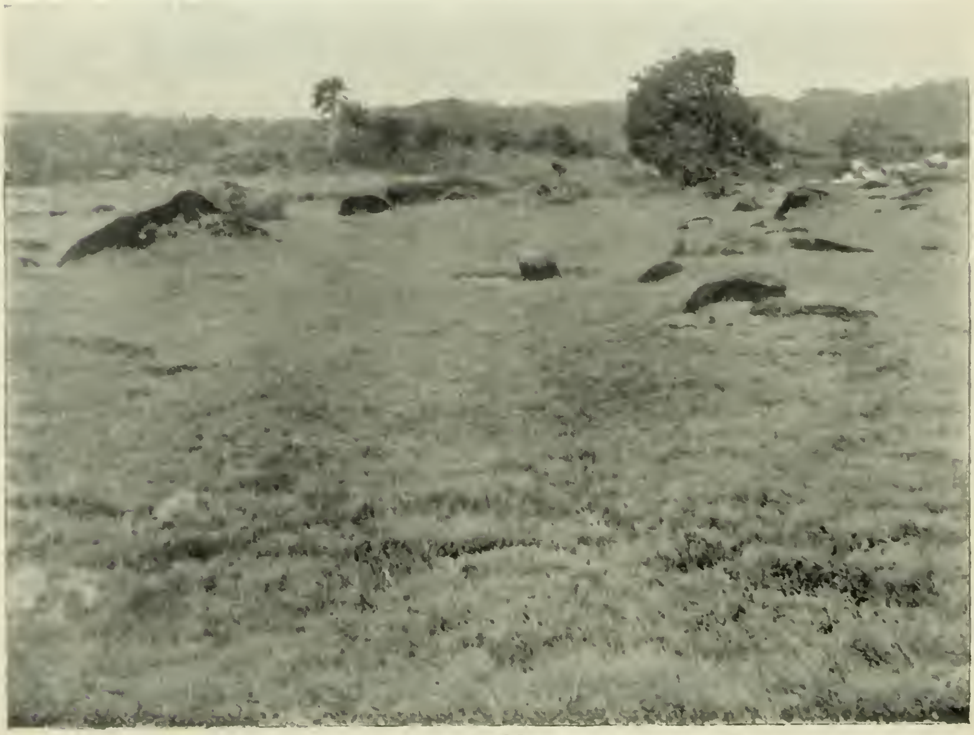

Plate No. 9.

Swampy flats between Kissy Road and Fourah Bay. Suitable breeding ground for *Anopheles* mosquitoes. Washing in progress in the distance.

BREEDING GROUNDS OF *ANOPHELES* MOSQUITOES.

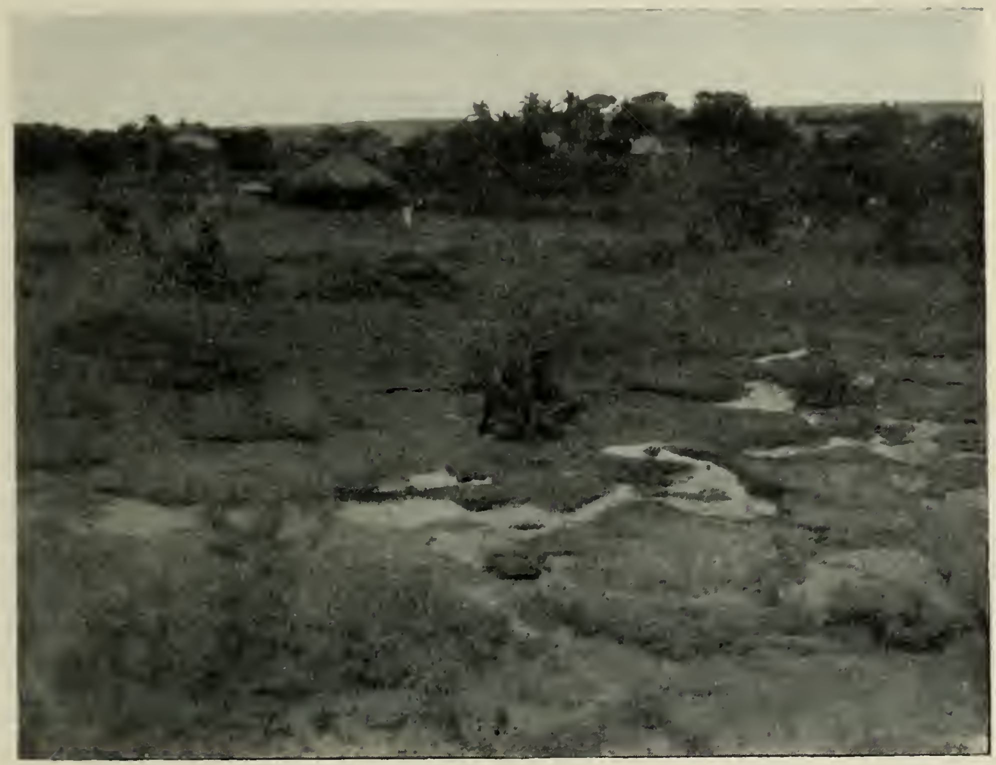

Plate No. 10.

Rock Pools on these flats; *Anopheles costalis* and *Stegomyia sugens* were breeding here.

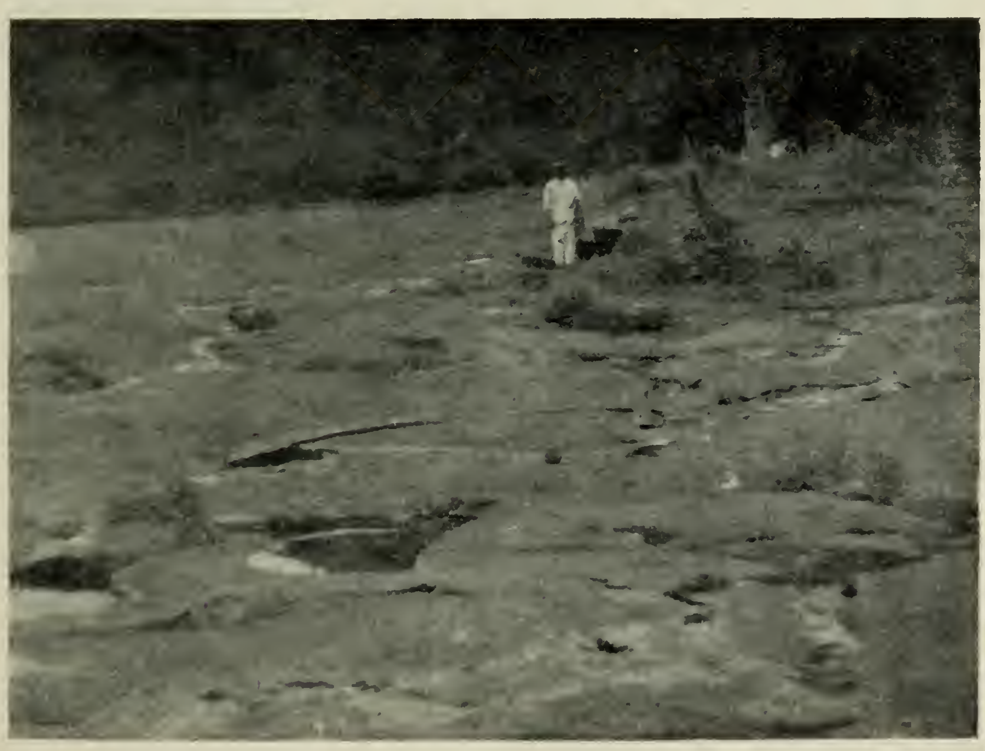

Plate No. 11.

Another view of the rock pools on the flats between Kissy Road and Fourah Bay.

Plate No. 12.

Details of the rock pools shown in Plate 10. Contained larvæ of *Stegomyia sugens* and *Anopheles costalis.*

Plate No. 13.

View of Kissy flats, near the Station. Breeding grounds of *Anopheles funestus.*

Kissy Road.

Rock pools in wayside gutter near the cemetery (*see* Plate No. 8, page 12):—

Anopheles (Pyretophorus) costalis, Lw. ... ... several.
Stegomyia sugens, Wied. ... ... numbers.

Flats between Kissy Road and Fourah Bay (*see* Plates Nos. 9, 10, 11 and 12, pp 12-14):—

Rock pools in shallow running swamp:—

Anopheles (Pyretophorus) costalis, Lw. ... ... numbers.
and probably *Myzomyia funestus*, Giles ... larvæ not reared.
Stegomyia sugens, Wied. ... ... swarms.

Kissy.

Rock pools in swampy lane:—

Anopheles (Pyretophorus) costalis, Lw. ... ... numbers.
Stegomyia sugens, Wied. ... ... swarms.

Pools in gutter by side of railway track:—

Anopheles (Pyretophorus) costalis, Lw. ... ... numbers.
Stegomyia sugens, Wied. ... ... swarms.

Shallow hard bottomed running swamps (*see* Plates Nos. 13, 14 and 15, pp. 14 and 16):—

Anopheles (Myzomyia) funestus, Giles. ... ... larvæ captured in twos and threes over large areas.

The following species were reared from larvæ taken from plants in different parts of Freetown:—

Cocoa Yam.

Stegomyia simpsoni, Theo. ... ... common.
,, *fasciata*, F. ... ... a few.
Culiciomyia nebulosa, Theo. ... ... occasionally. When present, usually in numbers.
Uranotænia ornata, Theo. ... ... common.
Eretmopodites dracænæ, Edw. ... ... a few.

"*Cock Hat*" *Tree* (*see* Plates Nos. 16 and 17, page 17):—

All the above species with the exception of *S. fasciata*.

Eretmopodites dracænæ, Edw. ... ... very common.

Banana.

Stegomyia simpsoni, Theo. ... ... not numerous.

Sarsaparilla.

Stegomyia fasciata, F. ... ... in numbers.
Eretmopodites dracænæ, Edw. ... ... a few.

Plate No. 14.
Another view; Kissy Village in the distance.

Plate No. 15.
Pools in the trickling swamps at Kissy Flats.

A PLANT WHICH AFFORDS BREEDING PLACES FOR MOSQUITOES.

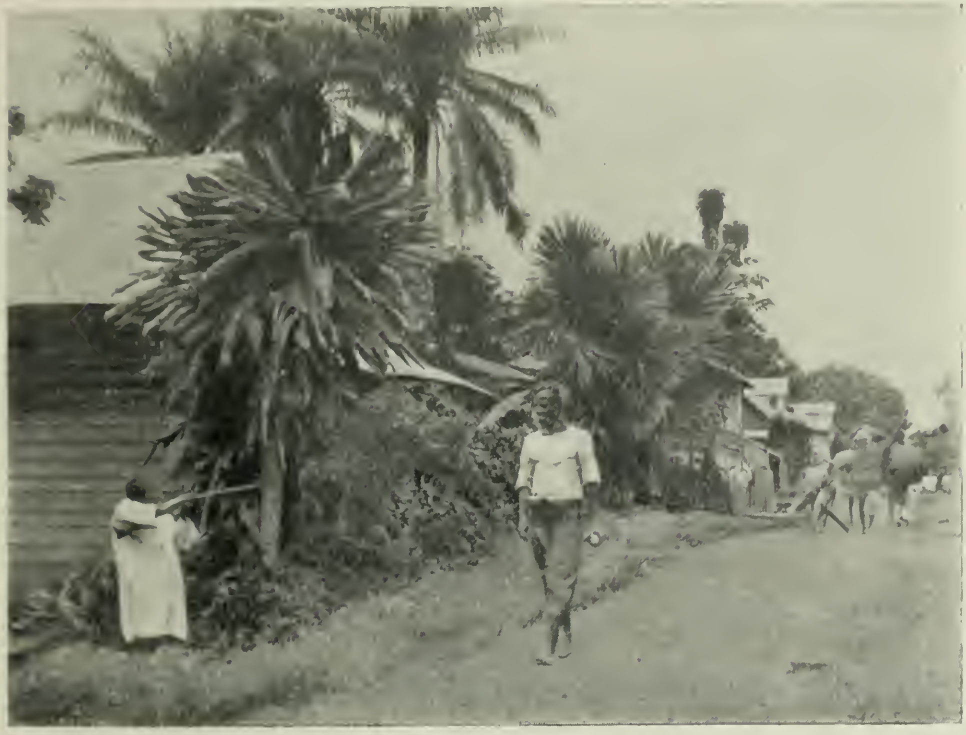

Plate No. 16.

" Cock Hat " Trees ; a species of *Dracæna*. The species in the foreground collects water in the central whorl and axils of the leaves. The stiffer leaved variety in the background holds no water.

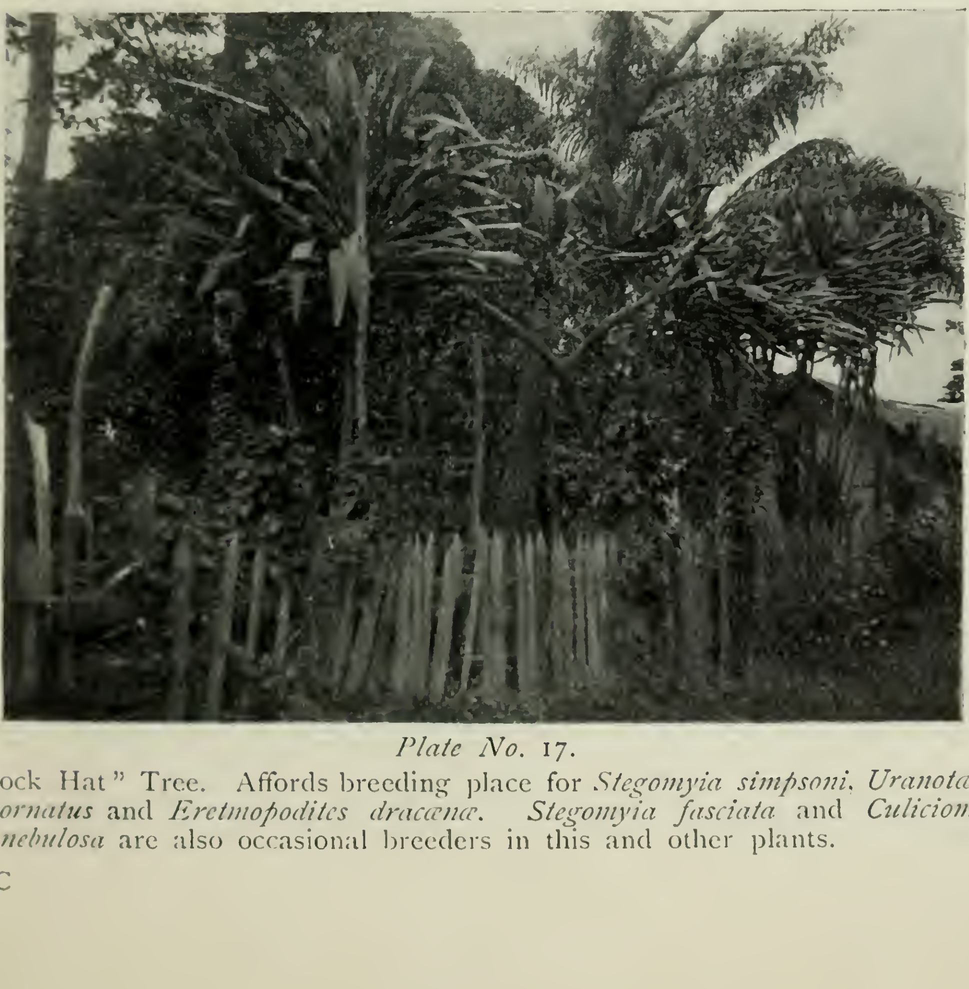

Plate No. 17.

" Cock Hat " Tree. Affords breeding place for *Stegomyia simpsoni*, *Uranotænia ornatus* and *Eretmopodites dracænæ*. *Stegomyia fasciata* and *Culiciomyia nebulosa* are also occasional breeders in this and other plants.

III.—BIONOMICS OF *S. FASCIATA.*

(*A*) EGGS.

General Notes.

The eggs of *Stegomyia fasciata* are small black spindle-shaped objects, which, as has been so frequently pointed out, are laid singly. They vary very considerably in size and shape, so that it is possible to pick out examples which in form and size closely approximate to the eggs of *S. sugens*, *S. simpsoni* or *S. luteocephala*, all of which differ from each other and, so far as the limited number of eggs of these species obtained is concerned, do not vary greatly individually in either size or outline. A noticeable feature of typical eggs of *S. fasciata* is that in one of their aspects they are slightly asymetrical, bow-shaped in outline, one side being flatter than the other.

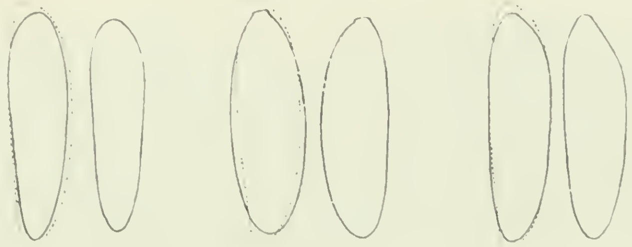

Fig. 1.

Outlines of the eggs of *S. simpsoni*, *S. sugens*, and *S. luteocephala*, showing the difference in shape and size between the eggs of these species, and an average egg of *S. fasciata*. The dotted outline is that of the egg of *S. fasciata*.

Size.—The measurements of the normal eggs of *S. fasciata* are about ·630 to ·650 mm. in length and ·160 mm. at the greatest diameter. Small eggs, frequently of stumpy form, are sometimes laid of scarcely more than two-thirds the normal size, while large eggs, measuring up to ·8 or ·9 mm. long and proportionately thick, are occasionally to be found. The diminutive eggs are generally, though not always, laid in batches by females all of whose eggs are small, and they frequently either do not hatch at all or show a high percentage of infertility. The giant eggs, on the other hand, are never laid in batches, but occur in twos and threes among eggs of more normal size and usually hatch. Pale, colourless, presumably immature eggs are occasionally found in batches of eggs which are of the normal colouration—these appear to be invariably infertile. Normally, however, all the eggs laid are either already dark when deposited or darken with great rapidity. The egg masses within the ovaries of the mosquitoes are usually colourless, but in many instances the ovaries of dissected mosquitoes were found to be already pigmented, usually of a bright brown. In England the number of white eggs seen after deposition suggests either that eggs were laid when less matured, or that the development of pigmentation is in some cases much less rapid, possibly owing to the lower temperature.

Surface Sculpturing.—The surface of the egg is covered with a delicate cellular reticulation formed by thin walls of chitin. From the centre of each cell space over the greater portion of the area arise hemïspherical bosses. At the blunt end the cellular reticulation is produced to form a low wall surrounding the micropylar area of the egg.

The Bosses.—The bosses within the cell walls are not merely structural irregularities of the egg wall, but are definite masses of a stainable substance, probably of a colloidial nature. Sections of the eggs within the ovary show that these bosses are apparently formed in pockets of a delicate inner membrane which enshrouds the egg.

The purpose of the structural features of the eggs of *Stegomyia* is usually assumed to be connected with the floating of the eggs, which are heavier than water and sink once they are freed from the surface film of water. In view, however, of the fact that surface structure of the shell alone would be quite sufficient, as in the case of other insect structures, to meet all the requirements connected with adjustment to the surface film, it seems not improbable that the bosses may serve an entirely different purpose to the cellular reticulation with which they are associated. This purpose may possibly be connected with the insulation of the egg from heated surfaces on which it rests, or, as seems more probable, with the ability of the eggs to resist desiccation.

In this connection it may be noticed that shrunken eggs, the sides of which are collapsing, often contain living larvæ, and, further, that such eggs will regain their full rotundity when placed in water, while after regaining their original shape they may still defer hatching to a later period. The suggestion is that by means of these bosses the egg is enabled to absorb moisture if wetted by rain or dew.

Situation where eggs are laid.—The eggs of *Stegomyia* are generally stated to be laid on the surface of the water. This may very possibly be accurate in the strict sense of the word, in that there is always a film of water present on which the egg is laid. Certainly no instance of an egg being laid on a dry surface has occurred with the thousands laid by captive females in the course of this research, and very few were ever laid in receptacles containing honey or syrup for the adults, even when there was no other liquid available for oviposition. As a general statement, however, it is misleading to say that the eggs are deposited on the surface of the water, as in the great majority of cases they are to be found on the wet margins of the receptacle containing the water, or of some partially submerged object, such as a leaf or twig. These situations suggest that they are either deposited on the margin itself—in some instances there was definite proof that such was the case—or on the water in such close proximity to the margin that capillary action on an evaporating surface inevitably strands the greater proportion of them.

The general position during oviposition would seem to be for the female to rest on the margin with her back to the water. In certain instances, however, the eggs are widely scattered over the water surface suggesting that they are deposited while the female walks on the water. All the evidence from the laying of eggs on wet filter paper points to the fact that they are deposited while she rambles about. It may be mentioned while dealing with this point that the eggs appear to hatch equally well whether resting at the bottom, floating, or attached to the margin beneath the surfare. Only a single instance of a female laying her eggs in a cluster on the surface of water was observed. In this case the female had thrust one or more of her legs through the surface film and was found lying on her side in a bogged condition alongside her eggs. The laying of eggs in clusters would seem to be of rare occurrence.

A few eggs, presumably only a small proportion of those laid on the water surface at its margin, sink at once. Others that have been laid or stranded on the steep or vertical sides of breeding pans are washed down, some sinking, while the remainder are restranded.

No doubt under natural conditions, where the range of circumstances is less restricted, the figures may be less one sided, but with

PHOTOGRAPHS OF EGGS OF *STEGOMYIA FASCIATA.*

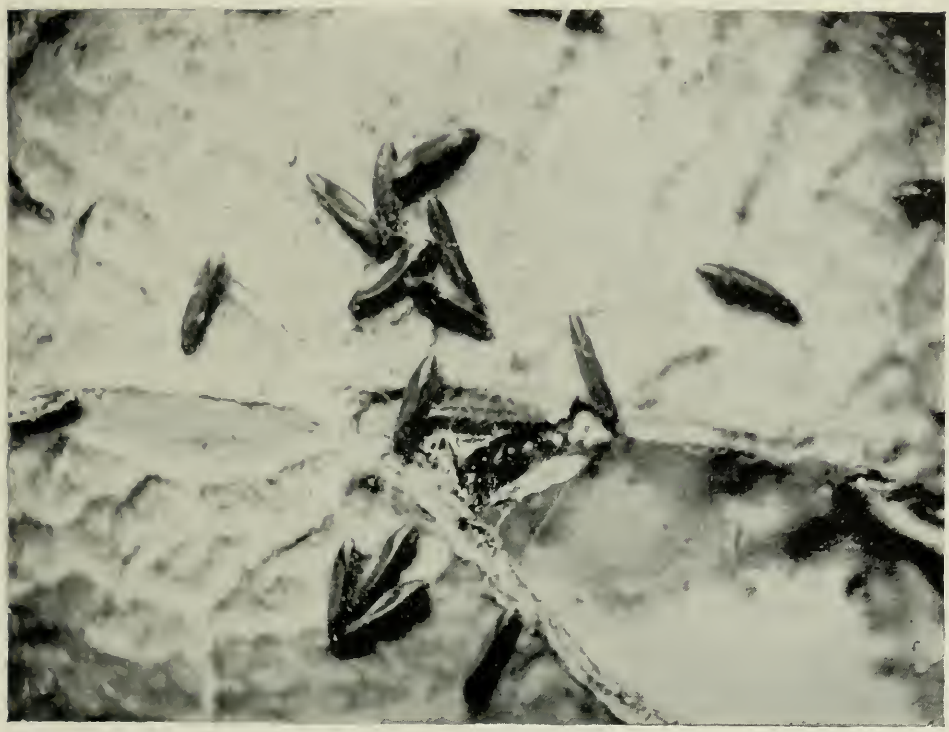

Photo by F. Noad Clark.]

Plate No. 18.

Eggs of *Stegomyia fasciata*, laid on leaf of Cottonwood tree. × 20.

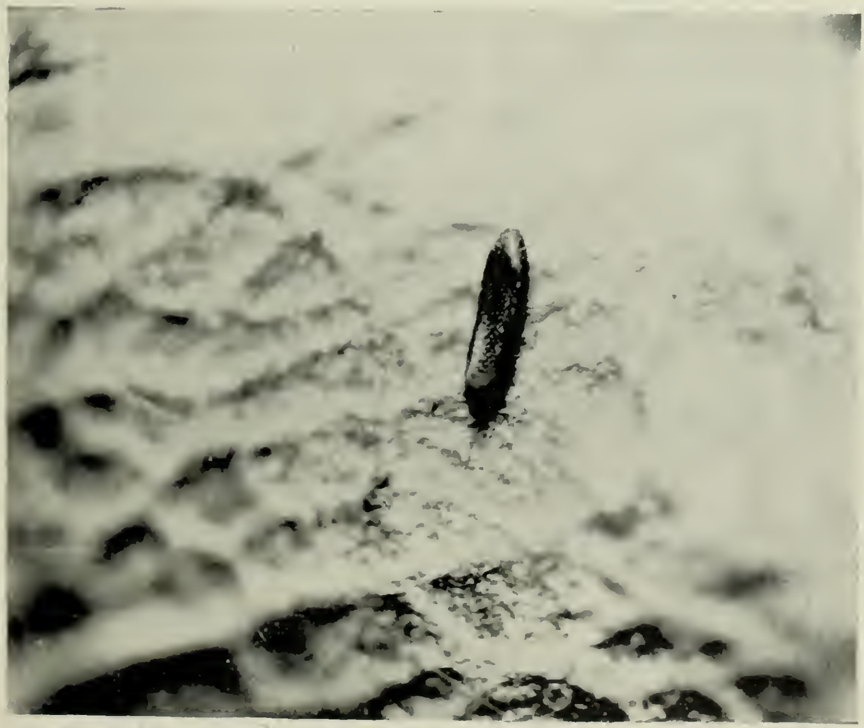

Photo by F. Noad Clark.]

Plate No. 19.

Egg of *Stegomyia fasciata*, laid on leaf of Cottonwood tree. × 40.

the methods adopted for rearing in this research by far the larger proportion of the eggs deposited were either laid, or stranded by capillary action, on the filter paper covering sides of the breeding pans above water level, at an estimate quite 90 to 95 per cent. Even with such badly adapted margins for stranding as tins would appear to afford, rust and dirt quickly produce an absorbent surface which acts almost as well as filter paper for the attachment or stranding of eggs.

Distribution after laying.—Eggs laid in the hollows formed in the beds of small streams, ditches, runlets, or guttering will tend to be washed downwards with each successive shower of rain and collect in the larger and more permanent pools, cisterns or water butts, as the case may be, as the attachment of the eggs is precarious in the absence of any other cement than that afforded by water scum, which, though firm enough during dry weather, is easily soluble in rain water. Eggs laid in sheltered water holes, in tree trunks, rock pools or tins are less liable to be distributed, but even in these cases a certain number will be laid on fallen leaves, some of which may be scattered during dry periods. With undisturbed eggs the gradual rise of water level during the onset of rain affords ideal conditions to induce hatching by gradually wetting and then immersing them in cool water, while at the same time the number of larvæ hatching tends to be automatically adjusted to the quantity of water.

Hatching.—The egg shell is ruptured by a clean transverse break at about a quarter of its length from the blunt micropylar end. Sometimes the cap falls completely off, but more frequently it is left slightly attached to or lying against the shell like a hinged lid. As the level of this clean cut corresponds with the position of the egg breaker, or more properly cutter, on the head of the larva, there can be little doubt as to the origin of the rupture in the shell which allows the larva to emerge. There are, however, some minor points connected with hatching which would seem to suggest automatic action on the part of the egg case and its contents apart from voluntary movement of the larva at the moment of its emergence.

In a number of cases eggs which had undergone long periods of dry storage, or had been submitted after long resistance of other methods to cooling, hatched, but yielded larvæ so feeble that they speedily died.

Eggs hatched under the influence of salt water show a considerable proportion which uncap although the larvæ do not emerge*. Again there is the existence of what may be termed a time zone system in the hatching of resistant eggs. They do not respond to the repetition of *stimuli* at short intervals in the same marked manner that they show if the repetition occurs after a considerable lapse of time. And further, as will be shown later in Experiment No. XVIII., there is evidence that the latent tendency to defer hatching, which is probably hereditary, may be considerably influenced by external conditions after incubation.

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* This also occurred to eggs that had been exposed to Formaline vapour for 24 hours.

In the light of these considerations it seems possible that the larvæ, which appear to be fully developed within 40 hours of the laying of the egg*, differentiate as regards the period when they perform the operation of incising the shell, those that have already performed this operation being ready to respond to external *stimuli* at once, while the others must delay until the necessary action is performed or defer indefinitely because the conditions have ceased to stimulate before the operation is completed.

It seems questionable to what extent volitional action on the part of the larva is responsible for the final uncapping of the egg. Sometimes the larva emerges with a "Jack-in-the-box"-like celerity, at others it will remain quiescent for a minute or so and then quickly and easily get free, or it may feebly and slowly struggle out.

Experimental Evidence Relating to the Hatching of Eggs.

The experimental evidence concerning the hatching of eggs is to a considerable degree bound up with questions of egg laying and fertility, which belong rather to the section dealing with adults. This is especially the case with the two preliminary Experiments, Nos. I. and II., which are included in this section, as they preface the whole of the experimental work dealing with hatching as well as that concerning egg production and fertilization; the chief value of these provisional experiments being in regard to the questions raised during their progress and the basis thus afforded for planning better controlled tests.

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* In Freetown at a temperature of 80° F.

Experiment No. III.

HATCHING OF EGGS LAID BY A WILD FEMALE OF *STEGOMYIA FASCIATA*.

A living female was found "bogged" on the surface of the water contained in a biscuit tin (at 26, Westmoreland Street). Her feet had broken through the surface film, and in this trapped position she had laid a batch of 59 eggs. In the light of later experience, the position in which the eggs were laid and their arrangement was most unusual; at the time it was thought that this was a normal instance of egg laying. The eggs were taken up on a slip of filter paper and a camera drawing of the arrangement of the eggs as laid was prepared. The batch was carefully submerged in a glass dish containing 200 c.c. of tap water.

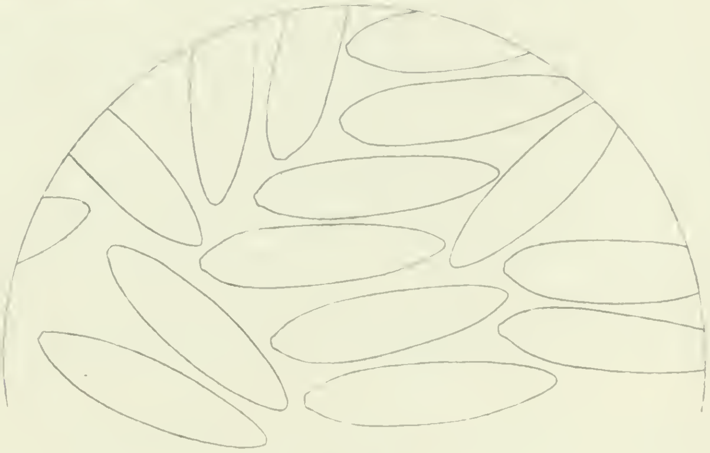

Fig. 2.

Date of laying.	Number of eggs.	Date of hatching.			
		September.		October.	
		27	28	5	12
24th September ...	59	35	3	**17**	**3**

Remarks and Notes.

The block figures indicate that hatching followed after a break in the continuity of the conditions.

On the 4th October the pan was refilled with fresh water, and on the 12th the eggs were lifted from the water for a space of four hours and allowed to dry; they were then re-immersed in the same water.

One egg remained unhatched; this was taken from the water on the 17th October, dried and then returned to the water. This was repeated on the 23rd October, and again for the fourth time a few days later. As the egg

Experiment No. I.

PRELIMINARY EXPERIMENT REGARDING THE LAYING AND HATCHING OF THE EGGS OF *STEGOMYIA FASCIATA*.

Two females and several males used.

The first female emerged 20th August, 1914; the second on the 1st September, 1914.*

The first male emerged 30th August, 1914; the second on the 4th September, 1914. Other males were placed with the females when these two died. Opportunities of feeding on human blood were afforded each night.

The eggs were collected each day from the wet filter paper on which the glass jar containing the mosquitoes was inverted.

All the eggs were submerged in water, none were allowed to remain floating.

Batches 1 to 9 were kept in separate glass dishes of various sizes, except the three laid on 12th, 13th and 14th September (*Nos.* 6, 7 and 8), these were in larger pans than the others and equal in size.

Batches 10 to 13 separated from each other in small glass dishes were all submerged in one large pan.

Batches 15 to 20 separated from each other in small glass dishes were all submerged in a second large pan.

Batches 21 to 30 kept in separate dishes.

†*Batch No.* 14. These 250 eggs, put down under date of the 22nd September, were collected from the rim of the jar. As no previous search for eggs had been made in this situation it is probable that this batch represented an accumulation covering several days. This situation was subsequently searched each day.

Dates on which eggs were laid.	Number laid.	Reference number.	Date of hatching.																										
			September.																October.										
			14	15	16	17	18	19	20	21	22	23	25	26	27	28	29	30	1	2	3	5	6	16	19	22	24	25	
4 September ...	11	1	...	...	1	2	...	...	...	...	...	...	...	...	...	...	**7**	...	...	...	...	...	...	...	...	...	...	...	1. The remaining egg failed to hatch and collapsed on drying.
6 ,, ...	36	2	...	...	...	...	...	1	...	2	1	1	...	...	...	...	**5**	...	...	...	...	...	...	...	...	...	...	...	2. The remainder dried on 10th October; all but one collapsed, this egg was opened and found to contain a larva.
7 ,, ...	16	3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	3. All failed to hatch during the month that they were kept in water and collapsed when dried.
9 ,, ...	112	4	2	2	1	3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	4. The remainder collapsed when dried on 13th October.
10 ,, ...	1	5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	5. Failed to hatch during the month it was kept in water and collapsed when dried.
12 ,, ...	49	6	...	...	49	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	6. For a whole batch of this size to hatch on the same day is very unusual.
13 ,, ...	62	7	...	...	1	4	**22**	**11**	**1**	...	...	...	...	...	...	...	**2**	...	...	...	...	...	...	...	...	...	...	...	7. The remainder collapsed when dried on 13th October.
14 ,, ...	67	8	...	...	...	...	...	...	1	3	...	1	...	...	...	...	**2**	**1**	**1**	...	...	...	...	**1**	...	...	...	...	8. When dried on 15th October all but one collapsed; this egg was returned to the water and hatched on the following day.
15 ,, ...	11	9	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	9. All collapsed when dried on 14th October.
17 ,, ...	41	10	...	...	...	...	...	...	...	1	1	...	2	1	6	...	**3**	**1**	...	...	...	...	...	...	...	...	...	...	10. The balance collapsed when dried on 14th October.
18 ,, ...	22	11	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	**1**	**2**	...	...	...	...	...	...	...	...	11. The remainder collapsed when dried on 14th October.
19 ,, ...	1	12	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	12. Collapsed before its removal from the water.
21 ,, ...	3	13	...	...	...	...	...	...	...	...	...	...	3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
22 ,, ...	†250	14	...	...	...	...	...	...	...	...	...	...	65	8	...	...	...	...	...	...	...	...	1	...	...	...	...	...	14. When dried on 25th October all the unhatched eggs except three collapsed. These three were returned to the water but failed to hatch; they were again dried on 9th November and immersed on January 15th, and failed to hatch, when dried for the third time they collapsed.
22 ,, ...	33	15	...	...	...	...	...	...	...	...	...	...	1	1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	15. The remainder collapsed when dried on the 16th October.
23 ,, ...	8	16	...	...	...	...	...	...	...	...	...	...	1	...	...	...	...	...	...	...	...	...	1	...	...	...	...	...	16. The remainder collapsed when dried on the 18th October.
24 ,, ...	86	17	...	...	...	...	...	...	...	...	...	...	...	...	6	74	1	...	...	...	...	...	...	...	...	**3**	...	...	17. The remainder collapsed after the second drying on the 23rd October.
26 ,, ...	12	18	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	18. All collapsed when dried on the 16th October.
27 ,, ...	8	19	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	5	...	...	...	...	...	...	...	...	...	19. As the remaining eggs failed to hatch and did not collapse they were stored on the 9th November, 1914, but were dead when opened in May.
28 ,, ...	83	20	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	5	24	6	...	...	...	**8**	**35**	...	...	20. After the third drying all the unhatched eggs but one collapsed, this egg resisted altogether five dryings and re-immersions; it was stored dry from the 9th November until the 30th December, when it was found to have collapsed.
30 ,, ...	1	21	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	1	...	...	...	...	...	...	...	...	
2 October ...	60	22	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	15	6	...	...	...	...	**20**	**1**	22. After the second drying there were 17 unhatched eggs which did not collapse. None of these hatched on the third immersion; they were dried and stored on the 9th November, 1914, but were dead when opened in May.
3 ,, ...	4	23	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	4	...	...	...	...	...	...	
4 ,, ...	4	24	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	24 to 30. These batches were kept immersed for three weeks, they were then dried; all collapsed.
5 ,, ...	2	25	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
7 ,, ...	3	26	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
8 ,, ...	12	27	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
9 ,, ...	7	28	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
10 ,, ...	4	29	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
11 ,, ...	31	30	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	
	1,040																												

* One female escaped on the 2nd October, the other died on 13th October.

Remarks and Notes.

Where the figures showing the number of eggs hatching are given in block type it is to be taken as an indication that the continuity of the conditions had been interrupted.

In the case of *Batch No. 7*, the eggs were shifted on the 17th September from a small to a larger pan filled with fresh water. On the 28th September the pans containing eggs of *Batches Nos 1 to 5* and *6 to 10* were re-filled with fresh water. The 3 larvæ that hatched from eggs of *Batch No. 17* on 22nd October did so within 15 minutes of their re-immersion in water after a second drying; the first drying and re-immersion having failed in its object.

The response of *Batch No. 20*, with 8 larvæ hatching on the 19th and 35 on the 22nd October, was apparently due to drying and re-immersion on the 18th and 21st October respectively. In this instance also most of the larvæ were free from the eggshell within 10 minutes of their being placed in water.

With *Batch No. 22* the hatching of the 20 larvæ that emerged on the 24th and 1 on the 25th October was apparently due to the drying of this batch on the 23rd, and its re-immersion on the 24th. Here again the majority of the larvæ were out within 10 minutes of the eggs being returned to the water.

E FEMALE WAS USED.

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E in regard to quantity and temperature of water.

DAT EGGS	AND NOTES.
	thout any apparent effect.
16 Sep	moval from the pan on 17th October.
17	
20	ed in 10 minutes, 1 later in the day, 1 the next day. Of the two g, while the other, after resisting five dryings and re-immersions, mber. It was then immersed and hatched within 30 minutes,
24	alance dried for the second time on the 23rd, and for the third d after the third drying. When re-immersed on 1st November, rs. On the 2nd November, the batch was dried for the fourth eggs hatched and the batch remained in water until the 21st water was put into an ice chest for an hour and a half; during n 80° F. to 73° F. 2 eggs hatched; remaining eggs dissected
24	sion Dried again on 24th October, and not immersed until 3 within 2 hours, and 3 more the following day. On the 3rd and re-immersed on the 4th; none hatched Dried for the ed without any eggs hatching. The batch was dried for the 0th January, when all the unhatched eggs were found to have
3 Oc	
5	mber, when the eggs were put in a cool, heavy rain for about 4, 3 and 26 hatched on successive days. The batch was kept ece of filter paper on which the eggs were laid was lifted from at was blowing; the batch was again immersed while the eggs remainder on the 24th December when cooled in ice chest.
9	mmersed; none hatched. They were again dried on 9th Nov- 2th May and found to be dead.
10	nmersed; none hatched. They were again dried on 9th n 12th May and found to be dead.

Experiment No. II.

EXPERIMENT REGARDING THE LAYING AND HATCHING OF EGGS BY *STEGOMYIA FASCIATA*, IN WHICH ONLY ONE FEMALE WAS USED.

The female emerged on the 10th September, 1914. Two males were kept with her; one emerged on the 11th September, and the other on the 13th September.

Opportunities for feeding on human blood were afforded each night from the 10th September onwards. The female died on the 27th October (life 47 days).

The eggs were collected each evening from the wet filter paper on which the glass jar containing the mosquitoes was inverted.

All the eggs were submerged in water. None were allowed to float.

Each batch when laid was placed in a solid watch glass and these were submerged in a large glass trough so that all the batches might have identical conditions in regard to quantity and temperature of water.

Date on which laid.	Number of eggs laid.	Reference number.	Date of hatching.																										Remarks and Notes.
			September.							October.							November.				December.								
			20	21	24	26	27	28	29	1	2	18	19	20	21	23	1	2	21	22	2	3	4	16	20	24	28	29	
																													The pan was refilled with fresh water on 6th October without any apparent effect.
September	8	1	1	2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	1. The remaining eggs were accidently destroyed after removal from the pan on 17th October.
	10	2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	2. All collapsed when dried on 17th October.
	16	3	...	...	1	1	...	...	...	...	...	...	11	1	...	...	...	...	...	...	...	...	...	1	...	...	...	...	3. Dried on 18th October, re-immersed on 19th, 10 hatched in 10 minutes, 1 later in the day, 1 the next day. Of the two remaining eggs, one collapsed after its third drying, while the other, after resisting five dryings and re-immersions, was stored dry from 9th November to 16th December. It was then immersed and hatched within 30 minutes, but the larva died before its first moult.
	32	4	...	...	...	3	...	1	4	...	...	...	...	...	...	1	10	...	2	...	...	...	...	...	...	...	...	...	4. Dried on 20th October, re-immersed on 21st The balance dried for the second time on the 23rd, and for the third time on 25th, 14 eggs retained their shape unaltered after the third drying. When re-immersed on 1st November, 4 hatched in 20 minutes and 6 more within 2 hours. On the 2nd November, the batch was dried for the fourth time and was not re-immersed until the 12th. No eggs hatched and the batch remained in water until the 21st November, when the pan containing the eggs and water was put into an ice chest for an hour and a half; during this period the temperature of the water fell from 80° F. to 73° F. 2 eggs hatched; remaining eggs dissected on 12th May and found to be dead.
	*99	5	...	...	...	26	4	4	4	1	1	...	...	...	...	...	23	3	...	...	...	...	...	...	...	...	...	...	5. Dried on 23rd October; none hatched on re-immersion Dried again on 24th October, and not immersed until 1st November; 3 hatched within 15 minutes, 23 within 2 hours, and 3 more the following day. On the 3rd November the batch was dried for a third time and re-immersed on the 4th; none hatched Dried for the fourth time on 5th November, and again immersed without any eggs hatching. The batch was dried for the fifth time on 8th November and stored until the 10th January, when all the unhatched eggs were found to have collapsed.
3 October	4	6	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	6. All collapsed when dried on 22nd October.
5 ,,	38	7	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	4	3	26	...	1	4	...	...	7. Dried on 5th November and stored until 2nd December, when the eggs were put in a cool, heavy rain for about half an hour: they were then immersed in water; 4, 3 and 26 hatched on successive days The batch was kept immersed until the 20th December, when the piece of filter paper on which the eggs were laid was lifted from the water and exposed to a very cool, dry wind that was blowing: the batch was again immersed while the eggs were still wet; 1 hatched within an hour, and the remainder on the 24th December when cooled in ice chest
	24	8	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	8. Immersed until 4th November, then dried and re-immersed; none hatched. They were again dried on 9th November and stored. Remaining eggs opened on 12th May and found to be dead.
,,	8	9	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	9. Immersed until 4th November, then dried and re-immersed; none hatched. They were again dried on 9th November and stored. Remaining eggs opened on 12th May and found to be dead.
12 ,,	4	10	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	1	...	...	...	...	1	...	...	10. Immersed until 8th November, then dried and exposed to rain on 2nd December; 1 hatched. Cooled on 20th December, by lifting from the water and exposing to cool, dry wind; cooled in ice chest on 24th December; 1 hatched. A second cooling by dry wind action on 28th December without effect. Dried and stored 31st December; examined April, when the remaining eggs were found to be dead.
13 ,,	18	11	...	...	...	...	...	...	...	...	...	1	...	...	...	...	...	...	...	...	4	...	...	...	...	...	2	1	11. Immersed until 8th November, then dried; exposed to rain on 2nd December, 4 hatched. Cooled in dry wind on 20th December; none hatched when re-immersed Again cooled in dry wind on 28th December. 2 hatched and 1 on following day. Remainder dried and stored 31st December; all unhatched eggs collapsed during 3 months' drying.
14 ,,	31	12	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	2	...	...	1	2	1	...	12. Immersed until 8th November, then dried, exposed to rain on 2nd December; 2 hatched on 3rd. Cooled in dry wind on 20th December, 1 hatched the same day and 2 when cooled in ice chest on the 24th. Again cooled in dry wind on 28th December, 1 hatched. Remainder dried and stored 31st December; examined 30th April, all unhatched eggs dead.
18 ,,	17	13	...	...	...	...	...	...	...	...	...	...	...	...	1	6	...	...	...	10	...	...	...	...	...	...	...	...	13. Immersed until 8th November, then dried and re-immersed 15th November; none hatched. On 21st November the pan of water containing the eggs was placed in an ice chest for an hour and a half; during this period the temperature fell from 80° F. to 73° F. All the remaining eggs hatched on the following day.
19 ,,	16	14	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	1	...	14. Immersed until 8th November, then dried and re-immersed; none hatched. On the 17th November an egg was opened and found to contain a fully formed larva to all appearances living, but it failed to make any movements when placed in water. Cooling in dry air on 20th December was without result, but after a repetition on 28th December 1 hatched. The remaining eggs were stored on 31st December. Remaining eggs opened 12th May and found to be dead.
21 ,,	23	15	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	2	...	...	1	...	...	15. Immersed until 8th November, then dried and re-immersed a week later; 2 hatched on 4th December (this was not a response to any break in the continuity of the conditions). Cooled in dry wind on 20th December; 1 hatched on 24th when cooled in ice chest. The second cooling in dry wind on the 28th December had no effect. Remaining eggs dried and stored on 31st December; examined 30th April, all unhatched eggs dead.
TOTAL ...	348																												

* The 99 eggs of Batch No. 5 put down under date of the 24th September, were collected from the rims of the jar; as no previous search for eggs had been made in this situation it is probable that this batch was an accumulation covering several days. This situation was subsequently searched for eggs each day.

did not hatch nor collapse, it was opened, and found to contain a fully developed larva in fresh condition, probably killed in the dissection of the egg.

Experiment No. IV.

EFFECTS OF EVAPORATION AND REPLACEMENT OF WATER.

A female that emerged 23rd–24th September was left for three or four days in a cage with a number of males and then segregated. Opportunities of feeding on human blood were afforded daily. The female died on 3rd Oct. Eggs were collected each day. All the eggs were submerged when in water. Very small pans were used, containing only 10 to 15 c.c. of water.

Dates on which eggs were laid.	Number of eggs laid.	Reference number.	Date of hatching.					
			October.					November
			3	6	7	12	21	11
30th September ...	12	1	8	2	...	...	1	...
1st October ...	7	2	...	...	1	1	3	1

Remarks and Notes.

1. The water was allowed to evaporate; by the 20th October the eggs were dry. Fresh water was added, and 1 egg hatched in two hours and a half. The remaining egg collapsed while in water.

2. The water was allowed to evaporate; by the 20th October the eggs were dry and fresh water was added. 1 egg hatched within twenty minutes, 2 more within five hours. The water was again allowed to evaporate, by the 10th November the two remaining eggs were again dry; 1 collapsed, the other was re-immersed and hatched within thirty minutes.

Experiment No. I. is concerned with the laying and hatching of eggs laid by two bred females which had the companionship of several males during the course of their lives.

Experiment No. II. is concerned with the eggs laid by a single female, but two males were placed with her in order to better the chances of all the eggs laid being fertilized.

The plan of putting batches of eggs in small dishes and immersing them in one large pan of water was extended in this experiment to the whole series, with a view of getting the conditions for all the eggs as nearly identical as possible.

Experiment No. III. dealt with a batch of eggs laid by a wild female; the opportunity afforded by this find was seized on as a means of checking results obtained when eggs laid by reared captives were used. As it turned out, the occasion was unique, but there is no reason to suppose that the eggs were in any way affected by the accident of their deposition.

Experiment No. IV. was initiated to contrast the effect of gradual evaporation of the water in which the eggs were submerged with the less natural methods employed to induce resistant eggs to hatch in Experiments No. I. and II.

The fact that 49 eggs forming batch 6 in Experiment No. I., which all hatched on the same day, were kept in a larger pan of water than batches 1 to 5 in the same experiment, raised the question of a possible relation between the size of the body of water and period of hatching.

The existence of such a relation was supported by the hatching of a large number of eggs of batch 7 (Experiment No. I.), following upon their transference to a larger dish of water. On the other hand, the addition of fresh water apart from any change in quantity was the apparent cause of batches 1, 2, 7, 8 and 10 hatching on the 29th September. A comparison with the hatching of eggs in Experiment No. II., when the quantity of water was much larger, and Experiment No. III., when it was equally large, pointed to the probability of the unanimous hatching of the eggs in batch 6 being a chance occurrence, while the partial nature of the response to either removal to larger pans or the renewal of water, together with the vagaries of hatching displayed by the batches immersed in the same pans of water in both Experiment No. I. and Experiment No. II., showed that the factors underlying hatching were probably of a complex nature.

The high percentage of apparently infertile eggs and the erratic nature of their distribution among the various batches of Experiment No. I. rendered it very doubtful if both the females had been fertilized.

It was an evident feature of all these early experiments that the incidence of hatching was dominated to a greater or lesser extent by external conditions, among which drying, the changing of water, and temperature conditions acted as *stimuli* on a certain proportion of the eggs only, wild laid eggs being affected as well as those laid in captivity.

The points raised which it seemed desirable to investigate further may be summarised in the following questions :—

(1) Does the quantity of water in which the eggs are immersed have any further influence on hatching ?

(2) Are all the eggs laid by a female capable of surviving drying; if a portion only, is the ability restricted to certain batches or evenly distributed?

(3) What is the effect on hatching of immediate as contrasted with deferred immersion; does the mortality rise in relation to the period of storage and is it affected by the humidity of the conditions of storage?

(4) Is temperature the stimulating factor which induces resistant eggs to hatch when dried and re-immersed, when they are transferred to fresh water, or when fresh water is added to that in which they are already immersed?

(5) For how long a period after impregnation is a female able to lay fertile eggs?

(6) How many females can a single male impregnate, and what percentage of the eggs of any one female will prove fertile after impregnation by a single male*?

To these may be added a question which practically belongs to this series, but was only formulated in response to results obtained in the conduct of the experimental work above outlined:—

(7) To what extent can resistance and the tendency to defer hatching be induced by external conditions after incubation?

In order to save time, owing to the probable death of some of the insects segregated for trial, most of the experiments were started in duplicate, and in some cases bulk experiments dealing with numbers of eggs laid in the general cages were performed dealing with the same or parallel points to support the results obtained when using single females.

It will be observed that information other than that directly aimed at was obtained in some cases; such by-products are commented on when dealing with the particular experiments involved.

The experiments dealing with Questions 1, 2, 3, 4 and 7 are included in this section, while those relating to 5 and 6 are placed in that dealing with the adults. An addendum to Experiment No. XLVI. (page 123), showing the detail of the number of eggs laid by one of the females and their dates of hatching, is included with the experiment to which it belongs in the adult section, although the evidence it affords is equally applicable to this.

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* The act of copulation is so rapidly and apparently frequently performed that it was not found practicable to limit the question to the result of a single pairing.

Experiment No. V.

EFFECT ON HATCHING OF SUBMERGENCE IN A SMALL OR LARGE QUANTITY OF WATER.

(*a*) Eggs Laid by a Single Female.

A female that emerged on 3rd or 4th of October was placed in a cage with a number of males. She was then segregated and afforded daily opportunities of feeding on human blood.

The eggs were collected daily and divided into two batches; these were placed in small glass dishes, one of which was submerged in a pan containing 400 c.c. of water and the other in a pan containing 4,000 c.c.

The water used was rain water that had been exposed to air and sun for a week and then strained through fine gauze.

All the eggs were kept submerged until the 8th of January, when they were dried and the sound unhatched eggs returned to the water.

Female died 22nd October.

Date of laying.	Number laid.	Number in each lot.	Reference number.	Date of hatching.																																Mortality.
				October.																November.																
				10	11	12	13	15	17	18	19	21	25	26	27	28	29	30	31	1	3	7	9	10	12	14	18	20	22	23	24	25	30	
7 October	62	31 small	1	16	1	2	2	...	...	...	...	...	...	...	...	...	...	...	...	...	1	...	...	...	...	...	...	...	...	1	4	1	1	6 per cent
		31 large	2	20	5	...	...	1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	4	...	...	...	..	3 per cent
14 ,,	48	24 small	3	...	...	...	...	...	1	4	...	...	...	...	...	...	1	...	...	...	...	...	...	...	...	...	...	4	14	...	...	...	...	Nil.
		24 large	4	...	...	...	...	...	3	13	2	1	...	...	...	...	1	...	...	...	...	...	...	...	...	...	...	1	3	...	...	...	...	Nil.
22 ,,	70	35 small	5	...	...	...	...	...	...	...	...	...	4	6	...	4	...	...	1	...	1	1	1	1	...	...	1	3	1	6	...	...	...	14 per cent.
		35 large	6	...	...	...	...	...	...	...	...	...	10	5	1	1	1	...	...	3	2	...	...	1	1	1	...	1	8	...	...	...	...	Nil.

Remarks and Notes.

1. One egg collapsed, one that did not was returned to the water, where it eventually collapsed. 2. One egg collapsed.
3. All hatched. 4. All hatched. 5. Five collapsed. 6. All hatched.

Experiment No. VI.

EFFECT ON HATCHING OF SUBMERGENCE IN A SMALL OR LARGE QUANTITY OF WATER.

(*a*) Eggs Laid by a Single Female.

A female that emerged on 4th or 5th October was kept in a cage with several males for two or three days; she was then segregated and given daily opportunities of feeding on human blood.

The eggs were collected each day and divided into two lots, which were placed in separate glass dishes. These were then submerged in the same pans as those used for Experiment No. V.

All the eggs were kept submerged until 8th January. When they were dried, the single unhatched egg of Lot 1 did not collapse and was returned to the water.

Date of laying.	Number laid.	Numbers in each lot.	Reference number.	Date of hatching.														Mortality.
				October.							November.							
				12	14	15	16	17	20	28	3	13	20	22	23	24	30	
9 October ...	14	7 (small)	1	1	...	...	...	...	...	1	...	...	...	2	1	1	...	14 per cent.
		7 (large)	2	...	...	...	...	...	3	...	...	1	...	3	...	...	...	Nil.
10 ,, ...	45	22 (small)	3	...	4	1	...	...	...	...	...	...	1	13	...	2	1	Nil.
		23 (large)	4	...	2	1	2	1	...	1	3	...	10	3	...	...	...	Nil.

Remarks and Notes.

1. The remaining egg subsequently collapsed.

The female disappeared out of jar on the 12th October. (Either eaten by ants or escaped, owing to some meddlesome person lifting the jar.)

Experiment No. VII.

EFFECT ON HATCHING OF SUBMERGENCE IN A SMALL OR LARGE QUANTITY OF WATER.

(*b*) EGGS LAID BY MORE THAN ONE FEMALE.

The batch of 192 eggs used for this experiment were laid in a cage containing a number of *S. fasciata* of both sexes. All the eggs were laid on the same night.

*The eggs were placed in small floating docks with a silk gauze bottom and allowed to sink or float as chance determined.

The water used was rain water that had been exposed to sun and air for a week and then strained through fine gauze.

Lot *A*, consisting of 94 of the above batch of eggs, was placed in a single dock floating in a small pan containing 100 cc. of water.

Lot *B*, consisting of 98 of the above batch of eggs, was distributed over 4 docks floating in a large pan containing 4,000 cc. of water.

LOT AND NUMBER OF EGGS.	DATE OF HATCHING.											
	OCTOBER.								NOVEMBER.			
	22	23	24	25	26	27	29	30	1	2	9	14
A. (small pan) 94 eggs ...	11	27	15	...	9	6	3	4	6	6	1	...
B. (large pan) 98 eggs ...	3	29	40	10	1	...	...	...	...	...	...	1

A. Total hatched at 30th November, 88 = 94 per cent.

B. ,, ,, ,, ,, 84 = 86 ,,

This experiment had to be abandoned at the end of November as a wild female got through the mosquito netting cover of the pan containing Lot *B* and oviposited in one of the docks.

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* This method was adopted in order to avoid the expenditure of the time required to sink so large a number of eggs, and also because of the essential difficulties of proving hatching if the eggs were simply allowed to scatter in a large pan. For full details of the method see description on page 178 and illustrations Plate 29 and Figure 27.

Experiment No. VIII.

EFFECT ON HATCHING OF SUBMERGENCE IN A SMALL OR LARGE QUANTITY OF WATER.

(*b*) Eggs laid by more than one female.

The 400 eggs used in this experiment were laid in a cage containing a number of both sexes.

The period during which the eggs were laid covered forty-eight hours.

The eggs were placed in floating docks.

The water used was from the pipe service at Freetown.

Lot *A*, consisting of 200 eggs, were placed in a single dock floating in a small pan containing 100 cc. of water.

Lot *B*, consisting of 200 eggs, were distributed in two docks floating in a large pan containing 4,000 cc. of water.

Lot and number of eggs.	Date of Hatching.																																	
	October.								November.																			December.	January.		February.			March.
	24	25	26	27	28	29	30	31	1	5	6	7	8	9	*10	11	12	13	14	15	16	17	20	22	23	27	28	10	24	28	11	16	18	5
A small pan 200 eggs ...	11	49	12	...	...	1	3	...	...	2	...	1	...	1	36	6	3	1	...	3	1	...	...	...	...	...	...	...	...	...	...	...	...	...
B large pan 200 eggs ...	1	10	45	3	2	1	8	1	7	4	4	3	1	2	5	7	2	8	2	8	14	2	1	2	4	2	2	1	1	2	2	1	1	1

A. Total hatched, 130. Mortality, 35 per cent.
B. ,, ,, 160. ,, 20 ,,

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* The morning of the 10th November was cooler than on the two previous days (80° F. on 8th, 78° F. on 9th, 76° F. on 10th). It is possible that this may have had some effect on the smaller quantity of water in lowering the temperature which was not operative in the large pan. It must be mentioned, however, that no similar occurrence was observed in the pans containing eggs of batches belonging to other experiments.

Experiments Nos V., VI., VII. and VIII. afford no very decisive answer to the first question. The most it is possible to say is that within the limits of the experiment the smaller quantity of water raised the percentage of mortality and was possibly responsible for some delay in hatching in Experiments Nos. V., VI. and VII., while Experiment No. VIII. tends to obscure the issue on this point. A very interesting fact clearly brought out by the series is, however, the length of the period which may elapse between the immersion of the egg and its hatching even when no dry period intervenes. In the Experiments No. V. and No. VI. periods of 40 to 50 days are shown, while in Experiment No. VIII. a few of the eggs waited 4 or 5 months before hatching.

Experiment No. IX.[*]

EFFECT ON HATCHING OF EARLY OR DEFERRED IMMERSIONS.

(*a*) EGGS LAID BY A SINGLE FEMALE.

A female that emerged on the 8th or 9th October was placed in a cage with several males and then captured whilst paired. She was then segregated and given daily opportunities of feeding on human blood.[†]

The eggs were collected each day (except when the number laid was very small) and divided into two lots, one of which were at once placed in a floating dock (I) while the other (D) were stored dry for a period of a month or more. They were then treated in the same way as the other lot.

The eggs were allowed to sink or float as chance directed.

[‡] Date of laying.	Numbers laid and in each lot.		Reference number.	Date of hatching.																																												Reference number.
				October.													November.																				December.										January.		
				16	17	19	20	23	24	25	26	27	28	29	30	31	2	4	5	6	7	8	9	10	11	12	14	15	16	18	19	20	23	24	28	30	1	11	12	14	15	16	17	18	19	21	7	20	
12 October ...	62	D 31	1																																							5	5	2	3	4	1			1
		I 31	2	31																																													2
13 ,, ...	48	D 24	3																																							4	13							3
		I 24	4	14	5																																												4
16 ,, ...	31	D 16	5																																							9	3							5
		I 15	6			4	9																																										6
19 ,, ...	58	D 29	7																																							1	24	1						7
		I 29	8					14	4	3	1																																				2		8
23 ,, ...	75	D 37	9																																							3	32							9
		I 38	10								30	1	1														1																						10
26 ,, ...	57	D 28	11																																								14							11
		I 29	12											3	12	2	5															1																	12
30 ,, ...	52	D 26	13																																								13							13
		I 26	14														18	1					1																										14
3 November	55	D 27	15																																														21	15
		I 28	16															2	4	0	4	2																									1		16
8 ,,	50	D 25	17																																														2	17
		I 25	18																					2	15	1																							18
12 ,,	30	D 15	19																																														11	19
		I 15	20																									10	1																				20
16 ,,	39	D 20	21																																														4	21
		I 19	22																											3	7	1	1																22
21 ,,	31	D 16	23																																														13	23
		I 15	24																															12	3														24
27 ,,	18	D 9	25																																														3	25
		I 9	26																																	2	6												26
8 December	48	D 24	27																																														1	27
		I 24	28																																			5	9										28
10 ,,	11	D 5	29																																														1	29
		I 6	30																																					1	2								30
12 ,,	46	D 23	31																																															31
		I 23	32																																							1								32

No more fertile eggs laid. The female was destroyed by ants on 10th January; life 95 days.

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[*] The companion experiment to No. IX. failed, owing to the premature death of the female.

[†] *See* Record of feeding and egg laying (page 34) for further details.

[‡] When only a few eggs were laid they were included in the previous or following batch.

Immediately Immersed Lots.

Some of the apparent mortality or infertility among the immediately immersed eggs must be attributed to the method employed of allowing them to either sink or swim in floating docks. While convenient in many ways for observation and effecting a very large saving in time and labour, this system is not without its drawbacks. Chief among these there is the fact that, after a few weeks' immersion it becomes impossible to follow up all the eggs individually, owing to the wood and gauze forming the docks becoming discoloured, while some of the eggs get washed into crevices at the junction of the wood and gauze.

The rusting of the tins used (in the absence of glass pans) was the cause of much discolouration. There may also have been a few actual losses owing to the gauze bottom of the docks rotting or fraying through with the lapse of time.

The docks containing the immediately immersed lots were taken from the water and dried in sequence between the 10th November and the 7th January in order to make room for those containing the later lots—the same water being used throughout. They were subsequently returned to the water, but it will be noted that only in the case of Lots Nos. 8 and 16 did eggs hatch after re-immersion.

Lots in which Immersion was Deferred.

No. 1. 5 eggs showed signs of collapse within three days of drying.
No. 3. 3 ,, ,, ,, ,, ,, ,, ,,
No. 5. 2 ,, ,, ,, ,, ,, ,, ,,
No. 7. 2 ,, ,, ,, ,, ,, ,, ,,
No. 9. (No record).
No. 11. 4 eggs showed signs of collapse within three days of drying.
No. 13. 4 ,, ,, ,, ,, ,, ,, ,,
No. 15. 5 ,, ,, ,, ,, ,, ,, ,,
No. 17. 4 ,, ,, ,, ,, ,, ,, ,,
No. 19.
No. 21.
No. 23.
No. 25. } (No record).
No. 27.
No. 29.
No. 31.

On the 14th December, Lots Nos. 1, 3, 5, 7, 9, 11, and 13 were examined :—

No. 1. 31 eggs present; 5 collapsed.
No. 3. 24 ,, ,, ; 4 ,,
No. 5. 16 ,, ,, ; 2 ,,
No. 7. 29 ,, ,, only partially examined; 23 were full.
No. 9. 36 ,, ,, ,, ,, ,, ; no collapsed seen.
No. 11. 28 ,, ,, ; 8 collapsed.
No. 13. 26 ,, ,, ; 4 ,,

All these seven lots were placed in floating docks on the 14th December, On the 19th January Lots Nos. 15, 17, 19, 21, 23, 25, 27 and 29 were examined:—

No. 15. 26 eggs present; all but 15 more or less collapsed.
No. 17. 8 ,, ,, ,, ,, 4 ,, ,, ,,
No. 19. 15 ,, ,, ,, ,, 6 ,, ,, ,,
No. 21. 14 ,, ,, ,, ,, 5 ,, ,, ,,
No. 23. 16 ,, ,, ,, ,, 13 ,, ,, ,,
No. 25. 9 ,, ,, ,, ,, 1 ,, ,, ,,
No. 27. 4 ,, ,, ,, ,, 2 ,, ,, ,,
No. 29. 5 ,, ,, ,, ,, 2 ,, ,, ,,

All these eight lots were placed in floating docks on the 19th January.

Unfortunately, the term "collapsed" was applied, during the early course of the experiment, to all eggs that showed marked signs of shrinkage, it being at that time considered that this was a certain forerunner of complete collapse, and that such eggs were infertile. Subsequent experience proved that this was not necessarily the case, as it was observed that partially collapsed eggs, when wetted may either hatch at once or recover their full rotundity and hatch later on.

The loss in numbers of Lots Nos. 17, 21, and 23 can only be accounted for on the assumption that they had been destroyed and eaten by *Psocidæ*, which had managed to get through the close fitting lids of the boxes in which the eggs were kept. These enemies had only penetrated into the boxes in which these three lots were kept.

FEEDING (ON BLOOD) AND EGG-LAYING OF *STEGOMYIA FASCIATA*.

Record of female used in Experiment No. IX.

Date of feeding and egg-laying.	Number of eggs laid.	Remarks *re* feeding.
12 October ...	62	Did not feed.
13 ,, ...	48	Fed.
14 ,, ...	...	,,
16 ,, ...	31	,,
19 ,, ...	59	,,
20 ,, ...	...	,,
23 ,, ...	75	,,
24 ,, ...	...	,,
26 ,, ...	55	,,
27 ,, ..	2	,,
28 ,, ...	...	,,
29 ,, ...	...	,,
30 ,, ..	52	,,
31 ,, ...	...	,,
1 November...	...	,,
2 ,, ...	7	Did not feed.
3 ,, ...	48	,, ,,
4 ,, ...	3	,, ,,
5 ,, ...	...	Fed.
6 ,, ...	...	Fed slightly.
8 ,, ...	47	Fed.
9 ,, ...	...	,,
11 ,, ...	2	Did not feed.
12 ,, ...	28	Fed.
16 ,, ...	39	,,
17 November...	...	Fed.
18 ,, ...	...	,,
20 ,, ...	...	,,
21 ,, ..	31	,,
26 ,, ...	7	,,
27 ,, ...	11	Did not feed.
28 ,, ...	...	Fed slightly.
1 December...	...	Fed.
3 ,, ...	...	,,
4 ,, ...	...	Bit, drew very little (if any) blood.
8 ,, ...	47	Fed.
9 December...	1	,,
10 ,, ...	11	,,
12 ,, ...	46	,,
14 ,, ..	...	Bit.
18 ,, ...	32	Fed.
19 ,, ...	...	Fed slightly.
22 ,, ...	...	,, ,,
23 ,, ...	3	Did not feed.

As only infertile eggs were being laid, a male was put in jar with the female.

Record of female used in Experiment No. IX.—continued.

Date of feeding and egg-laying.	Number of eggs laid.	Remarks *re* feeding.
24 December...	1	Fed slightly.
25 ,, ...	...	,, ,,
26 ,, ...	...	Bit.
27 ,, ...	20	Fed.
28 ,, ...	...	,,
29 ,, ...	2	Bit.
30 ,, ...	...	Fed.
1 January ...	5	Bit.
2 ,, ...	...	,,
3 ,, ...	3	Fed slightly.
4 ,, ...	4	,, ,,
5 ,, ...	...	Fed.
6 ,, ...	...	Fed slightly.
7 January ...	7	Did not feed.
9 ,, ...	...	...

As the male added on the 23rd December had died two fresh ones were added.

Date of feeding and egg-laying.	Number of eggs laid.	Remarks *re* feeding.
10 January ...	...	Fed heavily.
11 ,, ...	16	...

The female disappeared although both the males remained in the jar. I can only account for this by supposing that ants found their way in and carried off the female piecemeal.

10- to 15-minute opportunities of feeding on blood were afforded every night, but the dates on which egg-laying or feeding took place alone are recorded.

The last fertile egg was laid on the 12th December—63 days after separation from the males; at this time 712 eggs had been laid in 22 days. Allowing that eggs, when laid on following days, belong to the same batch (using this term to mean the ripe contents of the ovaries), this gives 15 batches of eggs within the fertile period.

The total number of eggs laid was 798, the last 87 being divided into 6 separate batches laid with intervals of more than a day between them.

The males introduced on the 23rd December and 9th January did not effect the fertilization of the eggs laid subsequently. During the period the males were with her, the female had the choice of feeding on honey as well as blood.

There is little doubt but that ants were responsible for the disappearance, as occasions in which legs, wings and other remains were left behind, as well as one instance when they were caught in the act, occurred during the course of the experiments. At the same time, I am disposed to consider that the female was nearly, if not quite, spent. It is possible, however, that if a chance of pairing had been given to her late in November, when signs of waning fertility at first began to show, that many of the late laid eggs might have proved fertile.

The evidence afforded by this experiment is, in a general sense, a sufficiently clear answer in the affirmative to Question (2), and the second portion of the question therefore falls.

In utilizing the evidence afforded by this experiment relating to the effect of immediate or deferred immersion (Question (3)), some preliminary precautions are necessary. Lots 17, 21 and 23 should be left out of account, as the ravages of book lice among the stored eggs not only reduced the numbers, but very probably, as their habit is, perforated others and killed the larvæ, leaving the shells intact. It will be better also to exclude all the batches subsequent to 26, as the general infertility of the December-laid eggs is very obvious.

Taking Lots 1 to 16, 19/20 and 25/26, we find that of the immersed eggs 199, = 82 per cent., hatched out of 244, while of those stored dry for a month or more before immersion 173 = 72 per cent., hatched out of 242, the period of dry storage being apparently responsible for 10 per cent. mortality.

Two batches, however, laid on the 19th and 23rd of October, require special attention in this connection. The eggs were deposited on wet wood (fragments of willow chip boxes) in place of the usual filter paper. It will be observed that the percentage hatching in these batches is high, 86 per cent. and 91 per cent., while they afford the only instance in which the stored eggs show a lower mortality than the portion of batch immersed immediately after laying. From this it would appear that the material on which the eggs rest may be a factor in their survival during periods of drought, a conclusion which fits in with the facts already noted in the introductory remarks (*see* page 19) regarding the recovery of shape by shrunken eggs.

*Experiment No. X.**

EFFECT ON HATCHING OF EARLY OR DEFERRED IMMERSION.

(*b*) EGGS LAID BY MORE THAN ONE FEMALE.

144 eggs were laid on the night of the 8th–9th October on filter paper in a cage containing numbers of *S. fasciata* of both sexes.

These were divided into two lots—*A*, 66 eggs, which were immersed on the 9th October, and *B*, 78 eggs, which were allowed to slowly dry and then stored in the animal house until the 17th November, when they were immersed, 39 days after laying.

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*In this and the following experiments, as well as in other cases where eggs were stored dry, woodlice and small insects, such as ants and *Psocidæ*, easily gained entrance to the eggs in paper envelopes, and, with the exception of the larger woodlice, to the wire gauze tubes as well. *Psocidæ* even penetrated into entomological boxes unless the lids fitted very tightly.

The surface of the filter paper on which the eggs were laid was often gnawed off in patches and numbers of eggs had disappeared. No evidence that either ants or woodlice intentionally destroy eggs was obtained, but, on the other hand, there is definite proof that *Psocidæ* do so. See account under "Enemies," page 59, Experiment No. XXII.

Lot *A*.

	Date of hatching.						Number of eggs which hatched prior to storage.	Percentage.
	October.							
	12	13	14	18	19	20		
66 eggs laid on the night, 8—9 October ...	3	20	11	1	2	1	38	57

On the 7th November, after 29 days' immersion, Lot *A* was taken from the water and stored dry in the Laboratory until the 22nd January (76 days). An examination showed 3 full and 16 shrunken eggs, the remainder having collapsed ; the eggs were then transferred to the mosquito house and placed under shelter. They were finally immersed on the 29th April after 173 days' storage without water. None hatched, and dissection showed that all the unhatched eggs were dead.

Lot *B*.

Was examined on the 17th November after 39 days' storage ; 9 of the eggs had collapsed. The lot was then immersed in water. After 3 days none of the eggs having hatched, the pan was placed in the ice chest, the temperature of the water falling within 2 hours from 81° F. to 73° F. ; 7 eggs hatched while in the ice chest. The pan was then kept at Laboratory temperature until the 4th January.

	Date of hatching.				Number of eggs which hatched after storage.	Percentage.
	November.			December.		
	20	22	23	21		
78 eggs laid on the night, 8—9 October ...	7	28	6	1	42	54

On the 4th January, after 48 days' immersion, the lot was removed from the water, dried, and stored in the mosquito house under cover until the 29th of April, when it was again immersed. None of the eggs hatched, and dissection proved that all were dead.

Experiment No. XI.

EFFECT ON HATCHING OF EARLY OR DEFERRED IMMERSION.

(*b*) Eggs laid by more than one Female.

493 eggs were laid between the 10th–14th October on filter paper in a cage containing numbers of *S. fasciata* of both sexes. These were divided into two lots—*A*, 197 eggs, which were immersed on the 14th October, and *B*, 196 eggs, which were slowly dried and then stored in the animal house until the 4th January, they were then immersed 82 days after laying.

Lot *A*.

	Date of hatching.						Number of eggs which hatched prior to storage.	Percentage.
	October.							
	14	15	16	17	18	20		
197 eggs laid 10—14 October ...	30	28	61	5	9	4	137	70

On the 7th November, after 24 days' immersion, Lot *A* was taken from the water and stored dry in the laboratory until the 22nd January (76 days). An examination showed 8 full and 24 shrunken unhatched eggs, the balance having collapsed ; the eggs were then transferred to the mosquito house and placed under shelter. They were finally immersed on the 29th April after 173 days' storage. After 14 days' immersion, during which period they were twice cooled, the unhatched eggs were dissected. *One living larva* was extracted, the remainder were dead.

Lot *B*.

Was examined on the 4th January after 82 days ; very many of the eggs were shrunken or collapsed. They were first put into a saturated atmosphere, and after 24 hours wetted by sprinkling and finally immersed ; 4 hatched within 2 hours.

	Date of hatching.									Number of eggs which hatched after storage.	Percentage.
	January.										
	4	5	6	7	10	14	15	16	17		
196 eggs laid 10—14 October. Immersed 4 January	4	17	1	2	1	3	18	3	2	51	26

They were dried on the 26th January and stored in the mosquito house, under shelter, until the 29th April, and were then again immersed after 93 days' storage. None of the eggs hatched, and dissection proved them to be all dead.

Experiment No. XII.

EFFECT ON HATCHING OF EARLY OR DEFERRED IMMERSION.

(*b*) Eggs laid by more than one Female.

517 eggs were laid between the 14th–16th October on filter paper in a cage containing numbers of *S. fasciata* of both sexes. These were divided into two lots, *A*, 254 eggs, which were immersed on the 16th October, and *B*,

263 eggs, which were dried, placed in a wire gauze tube and suspended in the covered pit in the mosquito house until the 18th November, 33 days after laying.

Lot *A*.

	Date of hatching.					Number of eggs which hatched prior to storage.	Percentage.
	October.						
	17	18	19	20	21		
254 eggs laid 14 – 16 October	70	87	27	17	1	202	80

On the 7th November, after 22 days' immersion, Lot *A* was dried and then stored in the Laboratory until the 22nd January (76 days). An examination showed 22 full and 22 shrunken unhatched eggs, the balance having collapsed; the eggs were transferred to the mosquito house and stored under shelter. They were then immersed again on the 29th April after 173 days' storage. *One hatched* on the 6th May and *one* on the 12th, both in response to cooling; but the larvæ died within a few hours of hatching. The remaining unhatched eggs were dissected; 2 were found to contain living larvæ, the remainder were dead.

Lot *B*.

Was examined on 18th November; it was found that the paper cover to the wire gauze tube was saturated with moisture, while the filter paper on which the eggs were laid was damp. 20 eggs had hatched owing to drops of water precipitated on the wire gauze having flooded single eggs or small groups of eggs; 13 eggs had collapsed; the remainder were immersed 33 days after laying.

	Date of hatching.										Number of eggs which hatched after storage.	Percentage of unhatched eggs at date of immersion.
	November.						December.					
	18	20	22	23	24	29	4	9	12	18		
Survivors of 263 eggs laid 14–16 October	35	15	82	19	6	3	1	1	1	1	164	68

A few of the eggs hatched within 3 minutes of their immersion, 18 larvæ were out within one hour and 35 within three hours. None hatched on the 19th. On the 20th November the pan was placed in the ice chest; in two hours the temperature of the water fell from 80° F. to 73° F., 15 hatched; the pan was then kept at Laboratory temperature until 3rd December, when it was placed in an incubator at 97° F. for two hours. Owing to an accident 18 unhatched eggs were lost. On the 4th January Lot *B* was dried and stored under shelter in the mosquito house until 29th April, when it was again immersed. No larvæ emerged, and dissection showed that all the unhatched eggs were dead.

Experiment No. XIII.

EFFECT ON HATCHING OF EARLY OR DEFERRED IMMERSION.

(*b*) Eggs laid by more than one Female.

580 eggs were laid between the 16th and 19th October, on filter paper, in a cage containing numbers of *Stegomyia fasciata* of both sexes. These were divided into two lots, *A*, 275 eggs, which were immersed on the 19th October, and *B*, 305 eggs, which were dried, placed in a wire gauze tube, wrapped in paper, and placed under dead leaves in the mosquito house.

Lot *A*.

	Date of hatching.									Number of eggs which hatched prior to storage.	Percentage.
	October.					November.					
	19	20	21	22	23	2	3	4	6		
275 eggs laid 16—19 October.	16	92	47	18	26	1	1	1	4	206	75

On the 17th November, after 19 days' immersion, Lot *A* was dried and stored in the Laboratory until the 22nd January (76 days). An examination showed 13 full and 16 shrunken eggs—the remainder of the unhatched eggs having collapsed. The eggs were transferred to the mosquito house and stored under shelter till the 29th April, when they were again immersed after 173 days' storage.

On the 2nd May, 1 hatched and died shortly after emerging.
,, ,, 4th May, 3 ,, one of which died.
,, ,, 6th May, 1 ,, 199 days after laying.

Dissection showed the remaining eggs to be dead.

Lot *B*.

Was examined on the 9th March, after 141 days' storage; the paper covering of the tube had been mostly eaten off—woodlice and millipedes were present on the outside of the tube, ants and *Psocidæ* in the interior. One of the four slips on which the eggs had been laid was taken out, the tube was recovered with paper and replaced under the leaves. Only 27 eggs were found on the slip in place of the 50 originally laid on it. Of these by far the larger number had already hatched, probably during heavy and prolonged rain in November or December; 2 eggs were full, 18 had hatched, and 7 had collapsed more or less completely. This portion of Lot *B* was immersed on the 9th of March and on two occasions between this date and the 20th of the same month, the paper with the eggs on it was lifted and cooled by exposure to draught.

1 hatched on the 20th—152 days after laying; a male was bred.
1 ,, ,, 24th—this larva died shortly after emergence.
1 ,, ,, 30th—this larva was dead when found.

All the remainder proved to be dead when opened.

On the 21st April, after 6 months' storage, the remaining 3 slips of Lot *B* were examined and then immersed :—

4 hatched when cooled on 23rd April, 186 days after laying.
1 ,, ,, ,, for the second time, on the 27th April, and died shortly after emerging.
1 hatched when cooled for the third time, on the 12th May, 205 days after laying.

Of the 305 eggs stored, 27 were found on the slip taken out on the 9th March, and 122 on the 3 slips examined on the 21st April ; in all 149 eggs. Of these, 43 had more or less completely collapsed ; 9 retained their shape and 97 had hatched while stored. 9 eggs hatched.

These experiments (Experiments X. to XIII.), while affording interesting general information relating to Question (3), fail to give a decisive answer to that portion of it which concerns a possible proportional relation between mortality and period of storage. This is due to the destruction by *Psocidæ* (book lice) of large numbers of the stored eggs in some of the experiments, and sufficiently numerous attacks in the case of others to throw doubt on the validity of the results. Such a heavy reduction of numbers was not foreseen, and in any case would have been difficult to guard against without entirely militating against natural conditions, as it would have been necessary to enclose the eggs in tubes or boxes with all but air-tight lids. Unfortunately, the cause of the reduction was not recognised until a late stage of the work, otherwise a careful examination of the stored eggs in Experiments Nos. X. and XI. might have allowed of some definite proportional results being arrived at. The excess in mortality, after 39 days' storage, of Lot *B* in Experiment No. X., is only 3 per cent., which, in view of the foregoing explanation, is probably devoid of significance.

In Experiment No. XI. the excess in mortality amounts to 40 per cent., which may be partly attributable to the lengthened period of storage, but in any case a very considerable allowance must be made for the destruction of eggs by these small enemies.

Experiment No. XII. was designed to contrast with No. X., the stored eggs being kept under very humid conditions. If allowance be made for a difference in the constitution of the eggs used, as shown by the different percentages which hatched of the unstored eggs in the two experiments, it would appear that storage under humid conditions, for the short period involved, affords no advantage.

Experiment No. XIII. Although such an extensive destruction of the stored eggs was disappointing, still a larger wastage than in the other experiments was to be expected, both on account of the longer period of time involved and also because the conditions of storage rendered the eggs not only more liable to the attacks of other insects but to be washed away by heavy rains as well. The reduced numbers are so small that but little reliance can be placed on any conclusion concerning these hatchings, more especially as it is uncertain to what extent the collapsing resulted from the attacks of book lice or from natural causes. If only the apparently sound eggs are considered, the result shows that 6 eggs out of 9 hatched (or 66 per cent.). This is probably far too high for a general survival rate under the conditions in the light of general experience, as apart from actual experiment, which suggests that long storage is certainly responsible for an increased mortality, apart from the attacks of enemies.

The Effect of Changes of Temperature.

The possibility of changes of temperature affecting the hatching of eggs was recognised at an early stage of the research. The preliminary trials which formed the basis for Question (4) suggested that a rapid fall of a few degrees Fahr. acted as a stimulus to induce the hatching of resting or resistant eggs, whereas an equivalent rise had little, if any, influence

The following experiments were then carried out, two plans being employed. In the first, which relates to cold only, eggs of many different stocks and layings which proved to be resistant under previous long continued immersion or repeated dryings and wettings, were cooled by one or other of the following methods:—

(1) By exposing the dry eggs to rain on a cool day.

(2) By placing the pans of water in which the eggs were immersed in an ice chest.

(3) By lifting the filter paper on which the eggs were lying from the pan of water and exposing it to rapid evaporation in a strong draught of dry air.

It is to be noted that in (2) and (3) the eggs had been kept immersed for several days before the trial, so that the larvæ had had opportunity of emerging right up to the time of the test. On the other hand, in method (1) the eggs, though already proved to be resistant, had been kept dry for a longer or shorter time before the trial.

In the second plan a large batch of eggs laid between the 23rd November and 7th December in a cage containing a number of *S. fasciata* was employed. One portion was stored dry while the other was immersed until all the eggs ready to hatch had done so.

The resistant eggs, which did not respond within a period of 17 days' immersion, were divided, one portion being submitted to a rapid rise of temperature by placing the pan containing them in an incubator at 95° F., while the pan containing the other portion was transferred to the ice chest.

The subsequent history of all the eggs that proved resistant to these conditions was, so far as possible, followed up.

Experiment No. XIV.

EFFECT OF COOLING ON RESISTANT EGGS OF VARIOUS BATCHES.

(1) By placing in rain on a cool, wet day (2nd December, 1914).

After remaining in the rain for about thirty minutes the eggs were placed in rain water and left out of doors for an hour. (The fall of the dry bulb of the hygrometer was less marked than that of the wet bulb on the 2nd December, 1914).

Reference to source from which the eggs were taken.	Date when the eggs were laid.	Number of eggs in the original batch as laid.	Number which hatched within 4 hours.	Number which hatched within 20 hours.	Notes regarding the previous history of the resistant eggs or the batches of which they formed part.
S. fasciata: —					
Exp. No. II., No. 7	5 Oct.	37	4	3	This batch of eggs had been immersed for 34 days before drying, but none of the eggs hatched.
Exp. No. II., No. 10	12 ,,	4	1	...	Immersed for 27 days before drying. No larvæ had emerged.
Exp. No. II., No. 11	13 ,,	18	4	...	Immersed for 26 days before drying. Only 1 larva had emerged.
Exp. No. II., No. 12	14 ,,	31	2	...	Immersed for 25 days before drying. No larvæ had emerged.
From Fertility Experiment: —					
B. II.	3 Nov.	35	4	...	Only 4 larvæ emerged during the period of immersion after laying, the resistant eggs had been dried and immersed several times without effect.
B. III.... ...	2 ,,	84	5	...	47 larvæ emerged within 4 days of laying; the resistant eggs had been dried and re-immersed on several occasions without effect.
B. III.... ...	5 ,,	80	1	...	65 larvæ emerged within 4 days of laying, and the resistant eggs had been dried and immersed several times without effect.
B IV.... ...	11 ,,	28	3	...	13 larvæ emerged within 4 days of laying, and the resistant eggs had been dried and re-immersed 3 or 4 times without effect.
C. I.	7 ,,	30	1	...	3 larvæ emerged within 6 days of laying; the resistant eggs had been dried and re-immersed several times without effect.
C. VII. ...	13 ,,	16	5	...	No larvæ had emerged within 10 days of laying, and the whole batch had been dried and re-immersed 3 or 4 times without result.

Experiment No. XV.

EFFECT OF COOLING ON RESISTANT EGGS OF VARIOUS BATCHES.

(2) By placing the pans of water in which the eggs were immersed in an ice chest.

Fall of Temperature from 81° F. to 73° F. Time in ice chest, about 2 hours.

Reference to source from which the eggs were taken.	Date when eggs were laid.	Number of eggs in the original batch as laid.	Number which hatched within 3 hours.	Number which hatched within 20 hours.	Number which hatched within 44 hours.	Notes with regard to the previous history of the resistant eggs submitted to the test.
S. lugens ...	27 Oct.	20	3	...	...	After several wettings and dryings had remained immersed for 6 days without any hatching.
S. fasciata :—						
Exp. No. X. ...	8/9 Oct.	70	7	...	28	The whole batch had been kept dry for 39 days before it was immersed. None of the eggs hatched in the 3 days that elapsed between immersion and cooling.
Exp. No. XII.	13/14 Oct.	263	15	...	82	The whole batch was kept dry for 33 days before its immersion. 35 larvæ emerged within 3 hours of the eggs being placed in water, but none hatched on the following day. On the third day after immersion the water was cooled.
Exp. No. II., No. 4	24 Sept.	32	2	...	...	After 4 periods of drying and wetting which followed the first immersion the resistant eggs had been immersed for several days prior to cooling.
Exp. No. II., No. 13	18 Oct.	18	...	...	...	After the first period of immersion the resistant eggs were dried; they had been again immersed for several days before cooling took place. *No effect.*
From Fertility Experiment :—						
B. III.	14 Nov.	84	...	...	...	Batch was immersed for first time on 18th Nov. when 27 hatched; on the 19th 31 hatched; none on the 20th; cooled on 21st. *No effect.*
B. IV.	8 Nov.	5	...	1	...	Immersed for 4 days prior to cooling.
C. I.	10 Nov.	31	6	2	...	,, 6 ,, ,,
C. I.	13 Nov.	18	1	...	...	,, 4 ,, ,,
C. I.	16 Nov.	37	...	1	...	,, 1 ,, ,,
C. IV.	13 Nov.	45	8	10	...	,, 4 ,, ,,
C. V.	15 Nov.	28	...	2	...	,, 1 ,, ,,

Experiment No. XVI.

EFFECT OF COOLING ON RESISTANT EGGS OF VARIOUS BATCHES.

(3) BY LIFTING THE FILTER PAPER ON WHICH THE EGGS WERE LYING FROM THE WATER AND EXPOSING TO A STRONG DRAUGHT WHEN THE HARMATTAN WAS BLOWING (28TH DECEMBER), THE EGGS BEING RE-IMMERSED IN THE SAME WATER BEFORE THEY WERE DRY.

Reference to source from which the eggs were taken.	Date when the eggs were laid.	Number of eggs in the original batch as laid.	Number which hatched within 1 hour.	Notes relating to the previous history of the eggs used.
S. fasciata :—				
Exp. No. II., No. 4	24 Sept.	32	...	Used in Cooling Experiment, No. XV.
,, No. 10	12 Oct.	4	...	,, ,, ,, No. XIV.
,, No. 11	13 ,,	18	2	,, ,, ,, No. XIV.
,, No. 12	14 ,,	31	1	,, ,, ,, No. XIV.
,, No. 14	19 ,,	16	1	A resistant batch, see notes to Exp. No. II., No. 14.
,, No. 15	21 ,,	23	...	A resistant batch, see notes to Exp. No. II., No. 15.
From Fertility Experiment :—				
A. II.	27 Oct.	9	2	Resisted long immersion and several dryings.
B. II.	3 Nov.	35	...	Used in Cooling Experiment, No. XIV.
B. III.	5 ,,	80	...	,, ,, ,, ,,
B. III.	2 ,,	84	...	,, ,, ,, ,,
B. IV.	11 ,,	28	1	,, ,, ,, ,,
C. I.	7 ,,	30	...	,, ,, ,, ,,
C. I.	29 ,,	21	1	Resisted a long period of immersion.
C. I.	3 Dec.	23	...	Resisted long immersion; 2 hatched when cooled on 24th December.
C. VII.	13 Nov.	16	2	Used in Cooling Experiment No. XIV.
C XIX.... ...	3 Dec.	47	1	Resisted 20 days' immersion.

Experiment No. XVII.

EFFECT OF CHANGES OF TEMPERATURE ON THE HATCHING OF EGGS OF *STEGOMYIA FASCIATA.*

The eggs used were laid on wet filter paper in a cage containing numbers of adults, between the dates 23rd November and the 7th December. The batch contained approximately 1,300 eggs*. About 600 of these (Lot *B*) were dried and stored dry from the 7th December until 24th March (107) days. The other portion (Lot *A*), about 700 eggs, were at once immersed in water without the filter paper on which they were laid being allowed to dry.

138 eggs (about 20 per cent.) hatched between 7th December and the 13th; the batch was, however, kept immersed until the 24th December, although no more larvæ emerged.

DETAILS OF HATCHING.

LOT *A*.	DECEMBER.							NUMBER OF EGGS WHICH HATCHED.	PERCENTAGE.
	7	8	9	10	11	12	13		
About 700 eggs laid 23rd November to 7th December...	8	69	42	11	3	4	1	138	about 20

Lot *A* was then divided into two portions: *A*1, containing about 420 eggs and shells; *A*2, containing about 250. *A*1 was placed in a pan with a portion of the water in which they had been immersed since the commencement of the experiment, and placed in an ice chest for 90 minutes—the temperature of the water falling from 80° F. to 74° F. The smaller lot, *A*2, was treated similarly as regards transfer, but the pan was placed in an incubator for the same period of time—the temperature of the water rising from 80° F. to 95° F. Eggs hatched as under:—

A1† "Cooled," within	$1\frac{1}{2}$	hours	102
,,	$2\frac{1}{2}$	,, when the temperature had risen again to 80° F. ...	4
,,	$3\frac{1}{2}$	,, ...	1
,,	24	,, ...	0
		Total ...	107 = about 25 per cent.

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* The difficulties of counting large numbers of these small eggs is considerable. Laid in scattered but closely approximating groups, the individual eggs are often in contact; accuracy in counting was only possible at an expenditure of time which was not available without sacrifice in other directions.

† 12 larvæ from each Lot, *A*1 and *A*2, were reared under precisely similar conditions:—
Of the "Heated," 7 attained maturity; mortality 41 per cent.
,, "Cooled," 11 ,, ,, ; ,, 8 ,,

A2[*] "Heated" within 1½ hours	... ...	2
,, 2½ ,,	when the temperature had again fallen to 80° F.	6
,, 3½ ,,	... ...	26
,, 24 ,,	... ...	6
	Total ...	40 = about 16 per cent.

On the day following the first test, both pans were put into the ice chest for 2½ hours; the temperature of the water fell from 80° F. to 73° F.; eggs hatched as follows:—

*A*1 "Cooled," within 2½ hours	... ...	2
2 days after	... ...	1
		3

*A*2 "Heated," within 2½ hours	... ...	13
5 days after	... ...	1
7 days after	... ...	1
		15

Adding these figures to those previously obtained, we find that 104 resistant eggs hatched in response to cooling—about 25 per cent. of the original Lot *A*1; of those submitted to heat only two actually emerged in response to the rise of temperature, about 1 per cent. of the original Lot *A*2, but that 53 hatched in response to falls of temperature subsequent to heating—about 21 per cent. This would seem to indicate that the temperatures employed had little, if any, effect, the actual stimulus being in both cases cooling in relation to the previous condition.

On the 4th January Lots *A*1 and *A*2 were lifted from the water and stored dry with Lot *B* until the 24th March (79 days). An examination of all three lots on that date showed that the number of eggs or shells in each lot had been reduced by 10 to 15 per cent., either owing to eggs becoming detached from the paper on which they were laid or, as I think, from the depredations of *Psocidæ*.

The three lots were immersed in separate pans on the 24th March, cooling in an ice chest being resorted to as opportunity occurred. The dates on which cooling took place are indicated by an asterisk. The fall in temperature of the water, which placing in the ice chest caused, was generally about 6° or 8° F.

On the 19th April the experiment had to be closed, owing to an accident.

At this point the influence of cooling is, however, sufficiently obvious for all practical purposes, although its effect differs in detail as regards Batch *A* (1 and 2) on the one hand, and Batch *B* on the other, probably because the less resistant eggs had not been weeded out of the latter batch by immersion prior to storing.

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[*] 12 larvæ from each lot, *A*1 and *A*2, were reared under precisely similar conditions:—
Of the "Heated." 7 attained maturity; mortality 41 per cent.
,, "Cooled," 11 ,, ,, ; ,, 8 ,,

Dates of hatching of Lot *B* and the balance of the resistant eggs of Lots *A*1 and *A*2.

Lots.	March.						April.														
	25	26	27	28	29	30	2	5	6	7	8	9	10*	11	12†	13	14	15	16	18‡	19
*A*1 (cooled) ...	...	10	...	...	1	2	1	...	...	1	...	...	7	3	41	12	3	1	...	6	3
*A*2 (heated)...	...	1	...	...	...	2	...	...	1	2	...	...	16	4	23	3	...	...	...	1	3
B (stored dry)	109	15	16	17	6	...	...	1	1	1	2	1	32	22	3	5	3	...	2	4	5

* On the 10th April the eggs hatched within 1 hour of the *commencement* of the cooling.

† The second cooling on the 12th April was slight, the fall being only 3° or 4° F. but it was long continued, lasting 5 hours.

‡ The third cooling on the 18th April was for 1 hour, the fall being from 85° F. to 74° F.

The final figures, although only approximate, are worth consideration. Of Batch *A*, 20 per cent. hatched on first immersion. Of the cooled resistant eggs (*A*1), 27 per cent. had hatched before they were dried and stored; 22 per cent. hatched subsequently, or, altogether, 49 per cent. Of the heated resistant eggs (*A*2), 22 per cent. hatched before and 22 per cent. after drying and storing; altogether 44 per cent.

The total number of Batch *A* which hatched is therefore 64 per cent., while of Batch *B* 41 per cent. hatched up to the time of the accident.

Of the totals, 36 per cent. of *A* had hatched in response to lowered temperature, and of *B*, 9 per cent.

The evidence afforded by these experiments seems fairly conclusive testimony that "cooling" is an important stimulus to hatching, and most probably is the effective factor when eggs are dried and re-immersed, or when fresh water is added to the pan containing the eggs.

It is to be noted that there is an underlying constitutional variability which withholds some of the eggs from hatching in spite of the stimulus. This appears to be of a zone-like character; that is to say, repetition of the stimulus at short intervals is less effective than if it is again applied after a greater lapse of time.

A further complexity arises from the fact that the zone-like character of this constitutional resistance to *stimuli*, together with the *resting habit as a whole*, may be suspended, with the exception of a small percentage of eggs, if certain conditions prevail at or about the period of incubation.

The experiment (No. XVIII.) performed to elucidate Question (7) was only planned and carried through at a late stage of the research when time did not permit of its repetition. The evidence it affords is, however, so definite and conclusive, with regard to the determining powers of drought or humidity (subsequent to incubation) either to bring the resting habit into action or to allow it to remain latent, that the only point remaining to be solved would seem to be whether the effect is invariable and relates to all eggs laid or if it is occasional only, relating possibly to a seasonal change in the constitution of the eggs. An answer on this last point would require a series of experiments carried out at, say, monthly intervals and continued over a period of 18 months to 2 years.

Experiment No. XVIII.

IMPORTANCE OF DRY OR HUMID CONDITIONS AS FACTORS IN INDUCING THE IMMEDIATE OR DEFERRED HATCHING OF EGGS.

A pan of water with two strips of filter paper, one end of which dipped into the water, while the other hung over the rim, was placed in a cage where females of *S. fasciata* were laying freely. Over 400 eggs were deposited on the filter paper during the night of the 10th April. The pan, with the eggs undisturbed, was then placed in a moist chamber (saturated atmosphere) for 50 hours to allow the eggs to incubate. A few of the eggs that were exactly on the water level or partly submerged, hatched.

The unhatched eggs were then divided into lots, one of which, Lot *A*, containing 192 eggs, was immediately immersed in a pan of water. 189 of the eggs hatched within 30 minutes. The 3 resistant ones, which were short, stumpy eggs, were kept immersed for 10 days; they were then dried and partly collapsed. They were again immersed for another 20 days and then dissected. Two contained dead larvæ and from the other a living larva was extracted.

The other portion of the batch, Lot *B*, containing 193 eggs, was allowed to dry on the bench. By the next day the number had been reduced to 168, owing to the attacks of *Psocidæ* (a species belonging to the genus *Clothilla*). Of this 168, 43 were more or less collapsed. (To a greater or less extent this collapsing may have been due to the *Psocidæ* perforating the eggs.)

Lot *B* was divided into :—

*B*1. Containing 86 eggs (15 of which were more or less collapsed). These were immersed after 24 hours' drying.

*B*2. Containing 82 eggs (28 of which were more or less collapsed). These were immersed after 7 days' drying.

The pans containing *B*1 and *B*2 were kept together and treated exactly alike as regards "cooling."

RESULTS.

*B*1.—86 eggs, 15 of which were more or less shrunken, were immersed after 24 hours' drying on the 14th April.

Lot *B*1.	Date of hatching.														Total which hatched.	Percentage which hatched.
	April.		May.				June.									
	15	28	12	19	20	21	5	10	11	12	14	16	18	19		
Immersed 14th April ...	18	12	4	...	1	...	1	2	8	1	11	4	5	5	72	84

Remaining eggs dissected 21st June and found to be dead.

*B*2.—82 eggs, 28 of which were more or less shrunken, were immersed after 7 days' drying on the 20th April.

Lot *B*2.	Date of hatching.														Total which hatched.	Percentage which hatched.
	April.		May.				June.									
	21	28	12	19	20	21	5	10	11	12	14	16	18	19		
Immersed 20th April ...	35	...	...	1	...	2	...	...	6	...	...	...	...	...	44	54

The remaining eggs and eggshells of Lot *B*2 were counted and carefully examined on the 5th July:—

59 had uncapped,
3 had collapsed,
8 apparently sound eggs were dissected:—6 contained *living* larvæ, 1 a dead larva, 1 was infertile.

The remaining more or less shrunken eggs contained dead larvæ.

The only feasible way of accounting for the discrepancy between the larval record and the shells is to suppose that, although a number of the eggs uncapped, the larvæ failed to get free, a by no means unusual occurrence, but the number (15) in this instance, is rather high, in relation to the size of the batch.

REMARKS AND NOTES.

On the 28th April and the 12th, 19th and 20th May, the pans containing the eggs were placed in an ice chest, the temperature of the water falling about 10° F.

On the 20th May, after the cooling, both lots were dried:—

Of *B*1,	31	eggs retained their full shape.
,, *B*2,	11	,, ,, ,,

Both lots were again immersed and put out of doors for the night to cool the water.

On the 3rd June both lots were again dried :—

Of *B*1, 36 eggs retained their full shape.
,, *B*2, 13 ,, ,, ,,

Both lots were again immersed.

On the 5th June and following days the pans were placed out of doors each evening so that the water might be cooled at night.

Experiment No. XIX.

AGITATION OF THE WATER AS A FACTOR IN HATCHING.*

Mitchell (1907) states that Dupree found agitation to be a factor in the hatching of ova of species of mosquitoes other than *Anopheles*, which deposit their eggs singly.† It was noticed that disturbance of water in which eggs were placed was coincident with the appearance of newly hatched larvæ of *S. fasciata*, but the phenomenon, being only of occasional occurrence, was rejected as a cause of hatching, in favour of the view that the agitation merely made larvæ that had been recently hatched show themselves, trial stirrings giving no consistent results. Upon reading Mitchell's statement, the following experiments were carried out :—

> A batch of eggs laid 4 or 5 weeks previously, which had been dry for 3 weeks, was placed in water—temperature about 73° F. 150 to 200 hatched within a few minutes, and by next morning 50 to 70 more larvæ were out. After an interval of 6 or 8 hours, during which none of the remaining eggs hatched, the water was agitated by shaking. 1 larvæ emerged and died. After a period of 20 hours, during which no more eggs hatched, the water was again agitated by a violent shaking. None of the eggs hatched. After 28 hours the water containing the eggs was placed out of doors to try the effect of cooling—none of the eggs hatched.

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* Experiments now in progress show that the contamination of water in which the eggs are lying has a marked effect on their hatching. Two trials performed since the MSS. went to press will serve to illustrate this clearly.

Into each of two glass pans, containing 100 cc. of tap water at 65° to 70° F., was placed one half of a slip of paper with upwards of 400 eggs of *S. fasciata* on it, there were approximately 200 eggs in each pan. To one pan (*A*) was added 3 cc. of water from a beaker which had been allowed to incubate at 97° F. for 48 hours with a fragment of human fæces in it. This water was thick and brown with the characteristic smell. To the other pan (*B*) nothing was added.

After 4 hours 14 larvæ had emerged in pan *A*, none in *B*.
,, 20 ,, 127 ,, were swimming ,, *A*, and 6 ,, *B*.
,, 70 ,, 136 ,, ,, ,, ,, *A*, ,, 42 ,, *B*.

The experiment was repeated, using fresh horse dung in place of human fæces.

No larvæ emerged in the first hour.

After 18 hours 148 larvæ had emerged from the eggs in pan *A* (contaminated water) and 11 in pan *B* (tap water only).

3 cc. of the manure water was then added to pan *B*. After 18 hours 159 larvæ had emerged, the stage of their growth suggesting that they had emerged within an hour or two of the addition of the manure water.

Further and more exact experiments with sterilized eggs and fluids are now being carried on by Dr. E. E. Atkin and myself. These, while supporting the above tests, suggest that the results are due to bacterial action. Under certain conditions eggs will not hatch while the fluid in which they are immersed remains sterile, but the infection of the fluid with bacteria causes the larvæ to emerge within a few hours.

† "Mosquito Life" (Page 26). Evelyn Groesbeeck Mitchell. G. P. Putnam & Sons, New York and London, 1907.

Time did not permit of the experiment being followed further. A second trial was made with a large batch of eggs laid 4 months previously. The batch was divided into two portions; both lots were immersed in beakers containing 200 cc. of water. Hundreds of larvæ emerged within a few hours. After 24 hours had elapsed, hatching had declined to a minimum and 6 hours later, no larvæ having emerged in either beaker, one of them was vigorously shaken while the other was placed out of doors for the night. Next morning 8 larvæ had emerged from the eggs in the cooled beaker: none had emerged in the one which had been shaken. The shaking and cooling were repeated 48 hours later, but none of the eggs hatched, and a later repetition was also ineffectual in both cases.

Time did not permit of the experiment being carried further. These trials cannot be considered as conclusive, but they suggest, if Experiments Nos. XIV. to XVI. are also taken into account, that agitation, if a stimulus to hatching, is less effectual in compelling larvæ within the eggs, which have not yet reached their hatching zone or period, to emerge, than cooling.

Experiment No. XX.

EXPERIMENTS RELATING TO THE PERIOD DURING WHICH THE EGGS OF *STEGOMYIA FASCIATA* RETAIN THEIR VITALITY WHEN OUT OF WATER.

The eggs laid on a filter paper or fallen leaves of the cottonwood tree were placed in wire gauze tubes or paper envelopes. In some cases, after the paper envelopes were placed in wire gauze tubes, these were again covered with paper in an attempt to exclude small predatory insects, such as ants, which could get through the mesh of the wire gauze. In spite of all precautions, however, the intended comparative statistical form of the experiments was vitiated owing to the ravages of one of the *Psocidæ* (book lice), a species belonging to the genus *Clothilla*. In many cases only a few out of hundreds of eggs stored remained to be tested, while in experiments where the destruction was less obvious it was impossible to form any estimate of the percentage of mortality due to long storage, apart from the attacks of these insidious little foes. As a consequence, the experiments only afford evidence of the period during which the eggs remain viable under various conditions, and not of the percentage which survive.

The tests were designed to fall into two Sections: (*A*) in which the eggs were exposed to the weather, and (*B*) where they were under cover. This plan had to be modified, however, to prevent the loss of eggs due to their being washed away by heavy rain, or to their hatching in small accumulations of water on the leaves or paper on which they rested.

During the dry season the tubes containing the eggs in Section *A* were not sheltered from sun or rain. An examination early in March showed that where paper coverings were used they had been eaten into holes by snails and millipedes, while both ants and *Psocidæ* had penetrated into the envelopes containing the eggs. In cases where no envelopes or paper coverings to the tubes had been used, many of the eggs had either fallen off or had been washed off by late rains, and in a number of cases eggs had hatched. With the oncoming of the rains further instances of eggs hatching on wetted paper occurred, and it was found necessary to cover the eggs with inverted tins to guard against further flooding.

In a few instances only was it possible to follow up separately the larvæ which hatched from different batches. Where time did not permit of this they were turned into a common breeding pan, from which large numbers of normal adults were reared. The tests dealing with the viability of the eggs were carried out in the same places as those dealing with the length of adult life. Charts giving curves of temperature and humidity for these situations will be found, with short descriptive notes, in the Experimental Series No. XXXV., on pages 64 and 92 to 101.

SECTION *A*.

Pen in Hospital Compound.

Eggs laid on leaves and filter paper, not placed in envelopes and tube not covered with paper—only a few eggs remained to be tested:—

Batch 1.—Laid during December; immersed 18th June. 1 hatched within 24 hours, two or three others emerged within the next two or three days; one of these died in its first skin shortly after emergence.

Vitality retained for 170 *to* 180 *days.*

Batch 2.—Laid during December, immersed 18th June. 1 hatched on the 18th, 1 on the 19th, 1 on the 20th and 1 on the 21st.

Vitality retained for 170 *to* 180 *days.*

One of the larvæ pupated on the 6th day after immersion; a male and a female emerged on the 8th day. Other specimens emerged later; the last to emerge was a male which took 11 days.

Eggs laid during January, on filter paper and placed in an envelope were also immersed on the 18th June, but failed to hatch.

Under Pen in yard at 26, Westmoreland Street.

Laid on leaves and filter paper, not placed in an envelope and the tube not covered with paper until commencement of the rains:—

Batch 1.—Laid during December, immersed 20th June. 2 hatched on the day after immersion; 1 larva died within an hour or two; three or four were hatched within the next few days.

Vitality retained for 175 *to* 180 *days.*

Batch 2.—Laid during December; immersed 23rd June. None hatched on the day of immersion, but several on the succeeding days.

Vitality retained for 175 *to* 180 *days.*

Two males and one female emerged within 7 days of the immersion of the eggs and two females on the 8th day.

Batch 3.—Eggs laid during January, on filter paper placed in an envelope which was kept in a paper-covered tube. Only 36 out of several hundred eggs remained. Immersed 23rd June; 7 hatched next day, 7 on the second and 2 on the third day.

Vitality retained for 145 *to* 150 *days.*

Development of larvæ not recorded.

On Ground in Mosquito House.

Laid during December on leaves and filter paper, not placed in an envelope and the tube not covered with paper until the commencement of the rains.

Immersed 13th July; one hatched the following day; no more emerged during the week that the trial was continued.

Vitality retained for 195 *to* 200 *days.*

Eggs laid on filter paper and placed in an envelope which was kept in a paper covered tube:—

Immersed 13th July.
1 hatched within 24 hours.
2 ,, ,, 48 ,,
4 ,, ,, 72 ,,
1 ,, ,, 120 ,,

Vitality retained for 165 *to* 170 *days.*

Eggs laid during December on filter paper and placed in envelopes, but not placed in tubes or again covered with paper:—

(1) A batch of eggs laid during December on filter paper was placed in water for 24 hours and the eggs which *did not* hatch were dried and stored.

Immersed 20th July.
1 hatched within 2 hours.
7 ,, ,, 48 ,,
2 ,, ,, 72 ,,
2 ,, ,, 120 ,,

Vitality retained for 200 *days.*

(2) Laid during December on filter paper and stored in an envelope without previous immersion.

Immersed 20th July.
53 hatched within 2 hours, 7 larvæ died within a few hours of hatching.
165 ,, ,, 24 ,, , 12 larvæ died within a few hours of hatching.
52 ,, ,, 48 ,, , 4 larvæ died within a few hours of hatching.
24 ,, ,, 72 ,,
3 ,, ,, 96 ,,
2 ,, ,, 120 ,,

Vitality retained for 200 *days.*

(3) Laid during December on filter paper and stored in an envelope without previous immersion.

Immersed 20th July.
20 hatched within 2 hours.
6 ,, ,, 24 ,,

Vitality retained for 200 *days.*

SECTION *B*.

Eggs Stored in Pit in the Mosquito House.

Laid during December on leaves and filter paper, not placed in envelope, tube uncovered :—

Immersed 6th July.
(1) 1 hatched within an hour.
Vitality retained for 187 *days.*

(2) Laid 7th to 14th December.
Immersed 6th July.
1 hatched within an hour.
4 ,, ,, 24 hours.
3 ,, ,, 48 ,,
1 ,, ,, 72 ,,
Vitality retained for 204 *days.*

Laid on filter paper during January, placed in an envelope which was kept in a paper covered tube.

Immersed 6th July, but failed to hatch.

Eggs laid on filter paper and kept in paper envelopes in the animal house until 31st December, then stored in the mosquito house pit :—

(1) Laid 20/23 October. Immersed 21st May.
About one dozen eggs of full and sound appearance remained.
2 hatched within 2 hours.
1 ,, after 17 days' immersion.
5 ,, ,, 20 ,, ,,
Vitality retained 210 *days.*

(2) Laid 24/26 October. Immersed 6th July.
Only 6 eggs of full and sound appearance remained out of about 100 stored ; there were many collapsed eggs, and signs of attack by *Psocidæ* were evident. None of the eggs hatched during a period of 10 days' immersion. On dissection, 1 living larva and 2 dead ones were extracted, the other 3 eggs collapsed while in the water.
Vitality retained 260 *days.*

(3) Laid during November. Immersed 25th July.
1 hatched within 48 hours.
1 ,, ,, 6 days.
Vitality retained 240 *days.*

Eggs laid on filter paper and kept in paper envelopes in the animal house until 31st December, then placed on ground in mosquito house under an inverted tin :—

Laid 29th October—5th November. Immersed 14th July.
1 hatched within 24 hours.
Vitality retained 250 *days.*

No more hatched during 10 days' immersion. On dissection a number of eggs were found to contain dead larvæ.

A tin containing leaves and filter paper on which eggs had been laid during January was left open on the Laboratory bench from January until the end of June ; it was then filled with water—11 adults were reared.

Vitality retained for 140 *days.*

An attempt was made to test the effect of exposure to the sun on eggs laid in tins and on leaves lying in tins. They were placed on the ground under a wire gauze pen in a part of the hospital compound which received full sunlight from 10 a.m. to 4 p.m. The eggs were laid in December and the exposure commenced on the 31st of that month, but the experiment came to an untimely end in January owing to the curiosity of the son (aged 5) of a German prisoner (his mother was a patient in the Hospital).

A second attempt was made after his departure in February. Three tins and a glass jar in which eggs had been laid during January, either on the sides or on dead leaves contained in them, were exposed. Water was poured into these receptacles on the 5th June.

Eggs hatched in one of the tins only, and several *Stegomyia fasciata* were reared by the middle of June.

Viability of Eggs of *Stegomyia fasciata* laid in Sierra Leone and brought to England.

Eggs from all these batches had been tested from time to time in Freetown and viability had been proved for from 4 to 6 months.

Laid 20th to 23rd Oct., 1914. Immersed 1st Sept., 1915. Failed to hatch.
,, 24th to 26th ,, ,, ,, ,, ,, ,,
,, 26th to 29th ,, ,, ,, ,, ,, ,,
,, 5th to 13th Nov. ,, ,, ,, ,, ,,

Laid 15th January, 1915. Immersed 16th Sept., 1915. Two hatched within 24 hours.

This was a large batch laid on filter paper, which had become so firmly attached to the tin sides of the breeding pan that on drying it could not be detached without scraping. The tin was cut up and portions with the undisturbed eggs on them were immersed. The two eggs that hatched only represent a small percentage of the eggs tested; certainly not more than 2 per cent.

Laid June-July, 1915. Immersed 16th September, 1915. Hatched freely.

Laid during July, 1915, on wood. Immersed 20th October, 1915. Hatched freely within 2 hours of immersion.

Laid during January on filter paper that became firmly attached to the side of a tin. Immersed 20th October, 1915. Hatched freely within 2 hours of immersion. *Vitality retained for 262 days.*

Experiment No. XXI.

TEMPERATURES WHICH EGGS OF *STEGOMYIA FASCIATA* ARE ABLE TO WITHSTAND.

It was found at Freetown that eggs easily survived a day or two at blood heat, 97° F., but more extreme tests were not practicable.

The following tests were carried out at the Lister Institute:—

First Test.

Eggs laid June–July, 1915, at Freetown on slips of filter paper and brought to England in a wooden box.

A slip was divided and one portion was kept in an incubator at 105·8° F. for 48 hours; the other portion was kept at room temperature, 65° to 70° F. On immersion the eggs on the slip placed in the incubator failed to hatch, while those on the control slip hatched freely.

Second Test.

A slip was divided into four, and the eggs on the several portions were submitted to the following conditions for 24 hours, the eggs submitted to heat were placed in a moist cell. At the expiration of the 24 hours all were immersed in water at about 64° F.

	After immersion at 64° F.		
	2 hours.	24 hours.	72 hours.
Incubator at 107·6° F.	...	...	...
Hot room at 96·8° F.	1 hatched	40 per cent. hatched.	70 per cent. hatched.
Incubator at 78° F. ...	A number	70 ,, ,,	75 ,, ,,
Cold room at 28° F. ...	1 hatched	20 ,, ,,	25 ,, ,,

Third Test.

A large batch of eggs laid during June and July in Freetown were placed in the cold room of the Lister Institute on the 18th September, and submitted to a temperature of 28° to 30° F. for 25 days; they were then placed in water at 65° F., subsequently raised to 75° F.; none hatched, but eggs of a control batch, kept at 65°–70° F, did so.

Fourth Test.

A batch of eggs laid on filter paper in London by specimens reared from Freetown eggs was divided into four lots; these were kept for 24 hours at 29°–30° F., 75° F., 95° F., and 102° F. respectively They were then placed in water at 65° F. and gradually raised to 75° F. after 20 hours' immersion.

Of the lot submitted to—

29°–30° F., 50 out of 69 hatched = 72 per cent. (one larva died).
75° F., 6 ,, 50 ,, = 12 ,,
95° F., 10 ,, 53 ,, = 19 ,,
102° F., none ,, 50 ,,

The pans of water containing the eggs were then allowed to cool down to 65° F. and examined after 4 hours. Of the lot submitted to—

29°–30° F., 6 had hatched = 9 per cent.
75° F., 20 ,, ,, = 40 ,,
95° F., none ,, ,,
102° F., 1 ,, ,, = 2 ,,

The pans were again kept at 75° F. and examined after 76 hours. Of the lot submitted to—

29°–30° F., none had hatched.
75° F., 2 ,, ,, = 4 per cent.
95° F., 1 ,, ,, = 2 ,,
102° F., 3 ,, ,, = 6 ,, (and were dead).

The pans were again allowed to cool to 65° F. and left. Of the lot submitted to—

29°–30° F., none had hatched.
75° F., 3 ,, ,, = 6 per cent.
95° F., 1 ,, ,, = 2 ,,
102° F., none ,, ,,

The pans were again placed at 75° F. and examined 20 hours later. Of the lot submitted to—

29°–30° F., none had hatched
75° F., 8 ,, ,, = 16 per cent.
95° F., none ,, ,,
102° F., 1 ,, ,, = 2 ,,

The pans were again allowed to cool to 67° F. and left for 4 hours Of the lot submitted to—

29°–30° F., none had hatched.
75° F., 1 ,, ,, = 2 per cent.
95° F., 3 ,, ,, = 6 ,,
102° F., 1 ,, ,, = 2 ,,

No further hatching occurred within a period of 4 days.

Result.

Temperature to which eggs were submitted for 24 hours.	Number of eggs tested.	Hatched within 128 hours.
29°–30° F.	69	56 = 81 per cent.
75° F.	50	40 = 80 ,,
95° F.	53	15 = 28 ,,
102° F.	50	6 = 12 ,,

Fifth Test.

Effect of heat *before* incubation.

A batch of eggs that had been laid not more than fifteen or sixteen hours was submitted to 97° F. for thirty minutes. The batch was then divided, half the eggs were subjected to a further rise of temperature to 114·8° F. for thirty minutes. Both lots were then stored dry for a few days at about 65° F. and then placed in water. All the eggs heated to 114·8° F. completely collapsed, and no larvæ emerged, while most of the control eggs hatched.

Experiment No. XXII.

ENEMIES IN THE EGG STAGE.

Under natural conditions there are probably many more enemies of this stage than appear when the eggs are stored under artificial conditions and protected against the attacks of vertebrates and the larger insects.

It is likely that some species of small bird may search out and devour the eggs in a similar manner to the Tits which destroy such multitudes of eggs of the winter moths in Northern Europe. Predaceous beetles and beetle larvæ possibly take toll, but no actual evidence of any of these attacks was forthcoming.

In captivity eggs stored out of doors were lost owing to the disturbing action of millipedes and woodlice, which gnawed the paper on which the eggs were laid, and to which they were somewhat loosely attached, but here again neither observation nor circumstantial evidence suggested differential action of such large enemies, which should have been apparent if the eggs were used as food. The same kind of loss to stored eggs occurs when they are laid on leaves, though this is probably chiefly due to bacterial action rotting the surface of the leaf in damp weather.

Ants might be expected to seek out and carry off the eggs, but all the evidence both general and experimental is entirely negative. It seems almost certain that the eggs must in some way be rendered distasteful to them, otherwise it is impossible to understand how the species of mosquitoes which rely on drought resisting eggs to tide over the dry season, could possibly survive in the face of the omnipresent activity of these insects in their search for food.

Indifference alone is hardly a sufficient reason for the passing over of eggs by a species of ants, one of which was detected in the act of laboriously carrying away a shred of aluminium foil.

The disappearance of eggs from boxes with such close-fitting lids that no ant could enter contrasted with the fact that they might be left uncovered on the Laboratory bench for days without interference, although ants were continually searching for and carrying away dead insects or any dried scraps of insects that might be about. This led to the following experiments, which prove conclusively that a species of *Psocidæ* (book lice), apparently the same species which occurs in Europe, was the enemy which had wrought such extensive destruction among the experimental eggs. It was found in nearly every situation, both indoors and out, which afforded sufficient cover. The *Psocidæ* themselves are attacked and utilized as food by a species of *chelifer*, or false scorpion.

Into a glass-topped entomological box, which contained remnants of dead mosquitoes and a numerous brood of *Psocidæ*, were placed two slips of paper on which eggs had been laid. On No. 1 the eggs had been laid and allowed to dry a month previously; at the commencement of the test there were 20 full eggs and 2 collapsed ones on it. On No. 2 the eggs had been laid and dried 5 months previously; examination showed that there were 14 full, 10 shrunken and 3 collapsed eggs on it.

On the following day, No. 1 had 13 full and 2 collapsed eggs on it, while No. 2 had 4 full, 8 shrunken and 2 collapsed eggs. There were also fragments of partly eaten eggs, and a careful examination showed that 2 of the apparently undisturbed eggs had small holes gnawed in them.

By the third day, in No. 1 there remained 4 full and 2 collapsed eggs, and in No. 2 but 1 full egg and some partially eaten remnants.

On the fifth day nothing but fragments of shell remained, which were shortly devoured, a special feature of the work of the *Psocidæ* being the completeness with which they clear up if time permits, not even the tiniest fragment of shell being left to afford evidence of the nature of the attacking agent.

The next test was to place a small batch of freshly laid eggs in the box. On drying there were found to be 22 full and 2 collapsed eggs. Two days later not a vestige of the eggs remained.

The third test was a comparative one. A large batch of freshly laid eggs was dried and the collapsed eggs removed, it was then divided into two portions.

Lot 1, consisting of 82 eggs, was placed in the box.

Lot 2, consisting of 67 eggs, was placed on the Laboratory bench, and left uncovered except on two occasions. On the 3rd of June a chip box was accidentally placed over the paper slip on which the eggs had been laid. On the 4th June the paper was turned over so that the eggs were between the paper and the bench. In both instances the accidental covering led to attack on the eggs.

LOT 1.

82 Eggs placed in Box containing numbers of Psocidæ.
Record of Reduction in Numbers.

MAY.			JUNE.											
26	28	31	3	4	5	6	7	8	9	10	11	12	13	15
82	77	76	68	61	54	47	43	28	17	8	5	3	1	0

LOT 2.

67 Eggs left exposed on Laboratory Bench.
Record of Reduction in Numbers.

MAY.			JUNE.											
26	28	31	*3	*4	5	6	7	8	9	10	11	12	13	15
67	67	67	45	42	42	42	42	42	42	42	42	42	42	42

* On the 3rd and 4th June the eggs were covered.

On the 15th June Lot 2 was transferred to the box.
After 24 hours only 20 eggs remained.
After 72 hours only 1 full, 1 shrunken and 1 collapsed egg remained.

(B) LARVÆ AND PUPÆ.

Experiments Concerning the Description and Quantity of Food needed by Larvæ of *Stegomyia fasciata*

General Notes.

The high mortality which occurs when the captured larvæ are simply placed in jars of water to rear, combined with the experience gained in breeding mosquitoes from the egg, show that an ample store of organic matter in the water to start with, or the addition of fresh food at intervals is essential. The description of organic matter used may vary within wide limits. Dead leaves, boiled white of egg, rice or other grain, dead insects, fæces, etc., all give good results provided the quantity is adjusted to the number of larvæ and quantity of water. Oily or fatty foods (such as cockroaches) may, however, easily cause a high or even a total mortality. So long as the water is free from oil or fat it may be very foul, cloudy or thick, and swarming with bacteria to an extent which suggests a broth culture. In the case of rice the water was so thick in some of the experiments as to resemble thin starch. Dead insects and other small animals, together with leaves, probably afford the chief natural sources of nourishment for the larvæ of *Stegomyia fasciata* and most of its relatives as well ; but apart from these, the fall of organic dust on any uncovered area would most likely suffice for a limited number of larvæ to attain the adult stage.

With *Stegomyia* and, so far as a limited experience goes, other *Culicine* mosquitoes, competition for the available food supply is very keen. To obtain a start before other organisms have had time to develop is an evident necessity if large broods are to be reared. Any quantity of fresh food added to standing water will not reproduce the favourable conditions which obtained at the initiatory filling of the breeding pool. This may be observed under natural as well as artificial conditions, and is probably largely due to the killing of the newly hatched larvæ by the larger ones, as pointed out by MacGregor (1915).

Stegomyia with its resting eggs, some of which are ready to hatch within a few minutes, and others within two or three hours of immersion, is better adapted to take full advantage of the late and early rains than species which have to deposit eggs requiring some 30 hours' incubation, after the pool has formed. Apart from such obvious competitors for food as tadpoles, ephemerid larvæ and those of species of *Chironomus* or other omnivorous water insects, a species of *Ostracoda* belonging to the genus *Cypris* is a highly successful competitor if it gets a start in breeding pans. Once it is established, few, if any, of the mosquito larvæ get sufficient food to finish their development.

Pans or jars, the sides of which become green with the growth of *Algæ*, are also very generally impossible for further breeding, but whether or no this is due to the *Algæ* or some secondary cause is undetermined.

The first moult is not passed by larvæ put into clean but unfiltered tap water. In the absence of added organic matter the larvæ seem unable to pass to the second skin until the death of some of their fellows provides the survivors with nutriment. If after a short but sharp period of starvation abundant food is given, the mortality may be low, and the larvæ reach maturity quickly, but produce small adults. On the other hand, if the period of scarcity is continued, the mortality is high, and the larval life may be unduly prolonged, possibly up to seventy days.

Under the most favourable conditions the larval life may be passed within four days, and males have completed their development within five days five hours.

The fact that larvæ breeding in water containing little organic matter produce small adults* has its practical side when cleanly tanks and covered cement-lined cisterns replace wooden butts or other dirty storage receptacles. The covers need to be of a smaller mesh, not less than 18 × 18 to inch, because in addition to the possibility of these dwarf females getting through the netting when seeking water to lay their eggs, there is always the risk of eggs that have been deposited in gutters being washed in through the netting and producing small sized specimens, which will squeeze through netting they would not otherwise pass in an endeavour to get out.

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* See also under "Enemies," page 79.

Protracted development or lagging may occur even in the presence of an abundant food supply; a few larvæ of any given batch will frequently lag behind their fellows. These lagging larvæ are often unhealthy and die, but they sometimes complete their development and produce normal adults after three or four times the usual period.

When considering the experiments some allowance must be made in respect of sex, the early males being usually a day quicker in their development than the females.

All the experiments with larvæ were performed in the Laboratory of the Colonial Hospital, and a Chart (No. 1) embodying the records of temperature and humidity from December, 1914, to July, 1915, is appended. A partial record of the maximum and minimum temperature and extremes in each week from September 11th to December 4th is set forth in Chart No. 9 on page 101.

Experiment No. XXIII.

QUANTITY AND NATURE OF FOOD REQUIRED.

200 cc. of tap water was put into each of three beakers.
To N.R. ·2 gr. of uncooked native rice was added.
To P.R. ·2 gr. of uncooked polished rice was added.
To W.E. ·2 gr. of hard boiled white of egg was added.

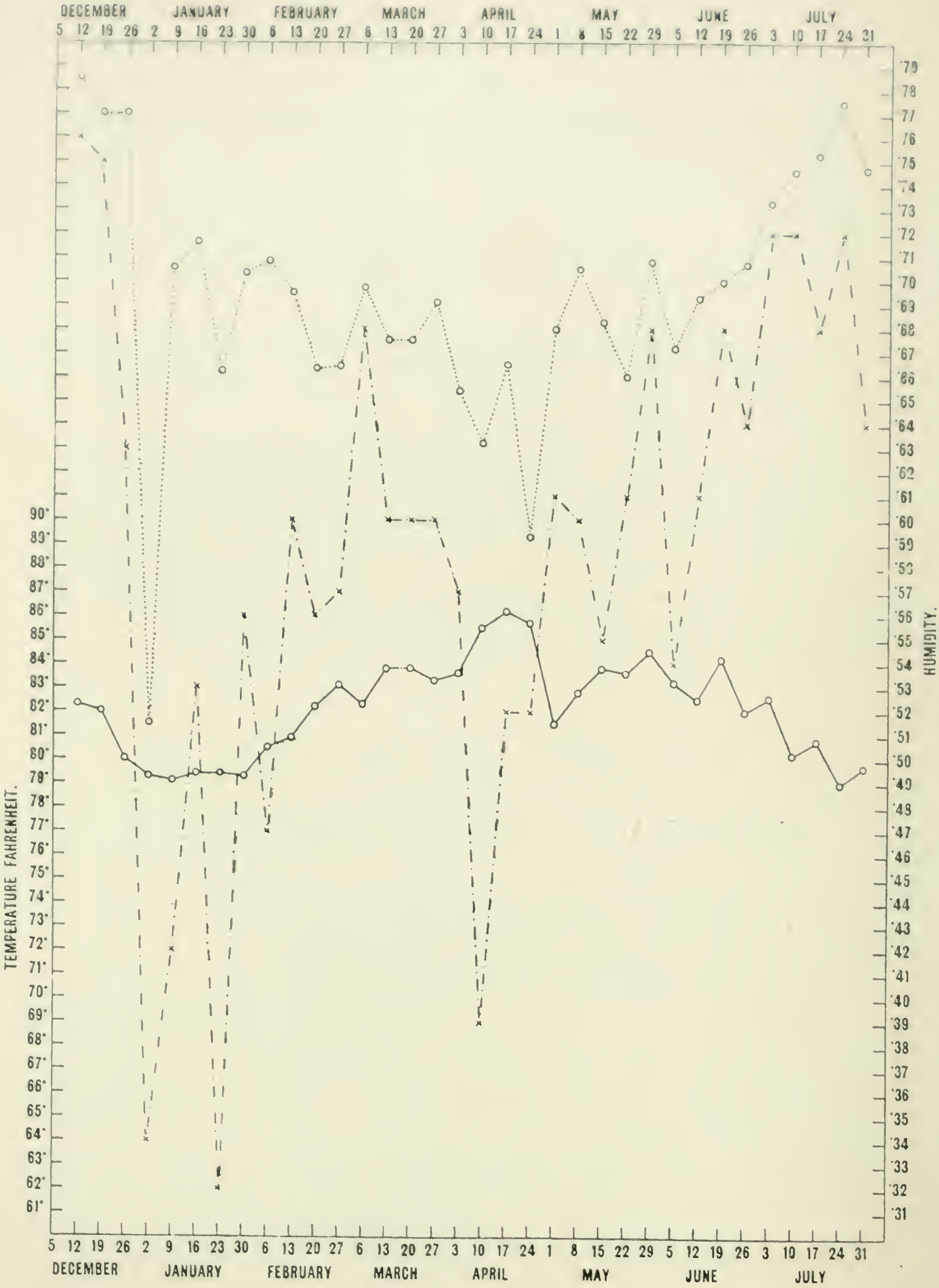
12 larvæ just out of their eggs (from a batch of eggs which had been dried and then re-immersed) were put into each beaker, and these were covered with thin cotton to exclude dust and small insects.

The temperature in the Laboratory during the course of the experiment averaged about 82° F.

2nd day after start—All the larvæ were of about equal size.
3rd ,, ,, ,, N.R. and P.R. ahead of W.E.
4th ,, ,, ,, P.R. most advanced; N.R. next, with W.E. a bad third, but all seemed equally healthy.
6th ,, ,, ,, P.R., 5 already in pupal stage; N.R., larvæ full-grown, but no pupæ; W.E., almost all the larvæ a moult behind.

CHART No. 1.

CURVES OF TEMPERATURE AND HUMIDITY IN LABORATORY AT COLONIAL HOSPITAL, FREETOWN.



Observations at 9 a.m. each day :—

Temperature ——————— Humidity, ...

Minimum Humidity record in each week — · — · — · — ·

FINAL RESULT.

Lot.	Number of adults reared.	Time occupied from hatching to adult state.			Mortality.	Remarks.
		Shortest.	Average.	Longest.		
N.R.	9 males, 2 females	9 days	10 days	12 days (1 female)	8 per cent.	...
P.R.	6 males, 6 females	8 days	9 days	11 days (2 females)	Nil.	...
W.E.	1 male, 6 females	26 days	31 days	42 days (1 female)	42 per cent.	...

The quantities of food used are not, of course, equivalent, as the water content of the white of egg will be much greater than that contained in the rice.

Experiment No. XXIV.

QUANTITY AND NATURE OF FOOD REQUIRED.

200 cc. of tap water was put into each of two beakers.

To P.R. 0·5 gr. of desiccated polished rice was added.

To W.E. 0·5 gr. of desiccated boiled white of egg was added.

16 larvæ, which had just emerged from eggs of the same batch as those used in the last experiment, were put into each beaker, and these were covered with thin cotton to exclude dust, etc.

The Laboratory temperature during the course of the experiment averaged about 82° F.

The larvæ in P.R. gained rapidly on those in W.E. at first, but subsequently the W.E. batch recovered most of the lost ground.

FINAL RESULT.

Lot.	Number of adults reared.	Time occupied from hatching to adult state.			Mortality.	Remarks.
		Shortest.	Average.	Longest.		
P.R.	10 males, 6 females	8 days	8 days	9 days (5 females)	Nil.	...
W.E.	10 males, 5 females	8 days	9 days	11 days (2 males)	6 per cent.	1 pupa died on the 13th day.

In this and the following experiments the food material was dried or desiccated before use.

Experiment No. XXV.

QUANTITY AND NATURE OF FOOD REQUIRED.

200 cc. of tap water was put into each of four beakers.

To O. nothing was added.

To P.R.O., 0·5 gr. desiccated polished rice was added.

To W.E.O., 0·5 gr. desiccated boiled white of egg was added.

To C.W.L., 1·0 gr. dried fragments of fallen cottonwood leaves was added.

20 larvæ just emerged from a large batch of eggs of mixed parentage, which hatched in response to cooling, were put into each. The beakers were covered with thin cotton to exclude dust, etc.

The temperature in the Laboratory at 9 a.m. during the course of the experiment averaged 79·4° F.

1st day :—No noticeable difference.

2nd day :—O., very little growth, no moults.

P.R., in second skin.

W.E., a few in third skin, but most in second skin.

C.W.L., far in advance of the others. Mostly well-grown in third skin, and a few approaching the third moult, but 2 larvæ still in first skin.

3rd day :—O., about half have grown, the remainder show no growth.

P.R., mostly full-grown in third skin, but a few only just past the moult.

W.E., mostly full-grown in third skin, but a few only just past the moult.

C.W.L., still ahead, but the gap between this batch and P.R. and W.E. is lessening. None have yet reached the fourth skin, but a few are preparing to moult ; 2 still lag, they are now in second skin.

4th day :—O., all are now making progress in the first skin.

P.R., all are well-grown in fourth skin, except 2, one of which is in the second skin and the other in the third skin.

W.E., all but 3 are well-grown in fourth skin ; of the laggards, 2 are in the third and 1 in the second skin.

C.W.L., are mostly marking time, but the backward larvæ are progressing ; one is in the second and the other in the third skin.

5th day :—O., still in first skin ; little appreciable further progress.

P.R., 3 have pupated ; 2 larvæ are still in third skin ; the remainder are full-grown.

W.E., 1 living and 1 dead pupa ; 2 larvæ in third skin ; the remainder full-grown.

C.W.L., 6 pupæ, 1 larva in third and 1 in second skin ; the remainder full-grown.

6th day :—O., still marking time in first skin.

P.R., 16 pupæ, 1 full-grown larva and 1 in third skin.

W.E., 6 pupæ, several larvæ dead ; the remainder full-grown.

C.W.L., 13 pupæ, 1 larva in third and 1 second skin ; the remainder full-grown.

7th day :—O., still no progress.
P.R., 18 pupæ and 2 larvæ, 1 in third skin.
W.E., 9 pupæ, two or three more larvæ dead.
C.W.L., 5 adults, 9 pupæ and 4 larvæ, 1 still in second skin.

8th day :—O., only 16 larvæ living; two or three of these have moulted and are now in second skin.
P.R., 13 adults, 2 pupæ and 2 larvæ, 1 still in third skin.
W.E., 4 adults, 6 pupæ and 1 larva.
C.W.L., 5 adults, 4 pupæ and 4 larvæ, 1 still in second skin.

12th day : O., 12 living larvæ, the largest backward in the second skin; the smaller ones still in the first skin.
P.R., finished.
W.E., 1 still in larval stage.
C.W.L., 3 still in larval stage; 1 only in third skin.

17th day :—O., 11 living larvæ, slight growth, but no moults since 12th day.
W.E., the last larva has now pupated.
C.W.L., 1 larva has pupated and an adult reared; the 2 remaining larvæ are both in fourth skin.

FINAL RESULT.

The larvæ of O. gradually died off, leaving a single survivor, which pupated after spending 48 days in the larval stage. A female specimen was reared. The larvæ did not kill one another, but lived on the decaying bodies of their fellows as they died.

Lot.	Number of adults reared.	Time occupied from hatching to adult stage.			Mortality.	Remarks.
		Shortest.	Average.	Longest.		
O. ...	1	50 days	...	...	95 per cent.	...
P.R. ...	16	8 ,,	8 days	9 days	20 ,,	1 larva died on 16th day.
W.E.	10	8 ,,	9 ,,	13 ,,	50 ,,	1 pupa died on 18th day.
C.W.L.	16	7 ,,	9 ,,	15 ,,	20 ,,	1 pupa died on 21st day.

When considering these results it is necessary to bear in mind three points :—

(1) the *quantity* of *organic matter* in relation to (2) the *quantity* of *water*, and (3) the number of larvæ in relation to both.

The result of O. affords further evidence of the result of insufficiency of food, but in a more acute form than W.E. in Experiment No. XXIII.

The mortality in P.R., as compared with the absence of deaths when the larvæ were fed on polished rice in the two earlier experiments, is probably not due to any scarcity of food owing to the larger number of larvæ present, but either to overcrowding or pure chance.

With W.E. in this experiment, the increased mortality, as compared with the use of the same food in Experiment No. XXIV., is also explicable as the result of overcrowding or chance.

With C.W.L., however, the result should be studied in relation to the use of fallen leaves of the cottonwood tree in the later experiments, the suggestion clearly being that the quantity of food used was barely sufficient for the number of larvæ, though the mortality may have been influenced, as in P.R. and W.E., by overcrowding.

Experiment No. XXVI.

ASSIMILABILITY IN ADDITION TO QUANTITY AND NATURE OF FOOD.

This experiment was planned with a view to ascertaining if speed of growth depended upon the assimilability of food apart from its nature. It was hoped also that some evidence of the action of bacteria or other micro-organisms in relation to this process might be obtained.

Into six beakers were placed the following food materials, all of which had been dried for a week in the desiccator:—

P.R. 1, 0·5 gr. polished rice.
P.R. 2, 0.5 gr. ,, ,,
C.W.L. 1, 0·5 gr. fragments of fallen leaves of the cottonwood tree.
C.W.L. 2, 0·5 gr. ,, ,, ,, ,, ,,
E.L. 1, 0·5 gr. fragments of fallen leaves of a species of eucalyptus.
E.L. 2, 0·5 gr. ,, ,, ,, ,, ,,

To beakers P.R. 1, C.W.L. 1, and E.L. 1, 200 cc. of tap water was immediately added.

All six beakers were covered with thin cotton to exclude dust.

The temperature in the Laboratory at 9 a.m. during the course of the experiment averaged 81·5° F.

After 24 hours a microscopic examination of the water was made by wet film.

P.R. 1, showed a few slowly moving rounded cells (? yeast).
C.W.L., a few bacteria and infusoria in each field.
E.L., no sign of any organism.

After 46 hours a further examination was made.

P.R. 1, showed numbers of bacteria + + + + and some of the slowly moving rounded cells.
C.W.L. 1, showed bacteria, numerous + + + +, also some infusoria.
E.L. 1, ,, ,, ,, + + + ,, ,,

200 cc. of tap water was now added to beakers P.R. 2, C.W.L. 2, and E.L. 2.

A large batch of eggs of mixed parentage was immersed, and from the larvæ which emerged within 90 minutes, 30 were placed into each of the six beakers.

The following history is general, the "lagging" larvæ are not individually noticed.

1st day :—P.R. 1, ahead of P.R. 2, but not to the extent of a moult.
C.W.L. 1, ahead of C.W.L. 2, but to a less marked extent than with P.R.
E.L. 1, very slightly ahead of E.L. 2.

2nd day :—P.R. 1, well-grown in second skin; P.R. 2, a full moult behind.
C.W.L. 1, rather less grown in second skin than P.R. 1.
C.W.L. 2, a moult behind C.W.L. 1; there is, however, less disparity between C.W.L. 1 and 2 than between P.R. 1 and 2.
E.L. 1 and 2 differ but very little from each other; they are both behind the others. None are past the first skin.

3rd day :—P.R. 1, mostly in fourth skin: a few almost full grown.
P.R. 2, more varied in growth; only a few in fourth skin.
C.W.L. 1, the advanced larvæ are about parallel with P.R. 2, but the proportion still backward in third skin is larger.
C.W.L. 2, quite noticeably behind C.W.L. 1, but only as a question of growth, not of moults.
E.L. 1, are slightly in advance of E.L. 2: both are behind C.W.L. 2.

4th day :—P.R. 1, all full grown, except a few laggards, which make very slow progress.
P.R. 2, in fourth skin, but not quite full grown; the laggards of this lot are, however, ahead of those of P.R. 1.
C.W.L. 1, not quite so advanced as P.R. 2.
C.W.L. 2, slightly behind C.W.L. 1.
E.L. 1 and 2, no noticeable difference; both lots behind C.W.L. 2.

6th day :—P.R. 1, 15 or 16 pupæ; one of the lagging larvæ is dead; all the remaining larvæ full grown in fourth skin.
P.R. 2, 8 or 10 pupæ; the lagging larvæ are now growing rapidly; all the others are full grown in the fourth skin.
C.W.L. 1, no pupæ; the larvæ are marking time as though food were failing.
C.W.L. 2, have quite caught up to C.W.L. 1, in fact, they look on the whole better fed, but there are no pupæ.
E.L. 1 and 2, are slightly behind the C.W.L's. in growth; they differ between themselves in that E.L. 2 shows a smaller range of variation in growth.

From this time onwards it became evident that the larvæ which were given leaves as a source of nutriment (C.W.L. 1 and 2, E.L. 1 and 2), had reached the limit of their food supply. Their record continues as a long-drawn-out period spent in marking time, with short spurts of growth as larvæ died, and supplied the survivors with fresh sources of nutriment.

FINAL RESULT.

Lot.	Number of adults reared.	Time occupied from hatching to adult stage.			Mortality.	Remarks.
		Shortest.	Average.	Longest.		
P.R. 1 ...	23	7 days	8 days	10 days	23 per cent.	...
P.R. 2 ...	29	8 ,,	9 ,,	11 ,,	3 ,,	...
C.W.L. 1	3	45 ,,	47 ,,	50 ,,	90 ,,	One larva lived until the 78th day, but failed to pupate.
C.W.L. 2	3	11 ,,	12 ,,	12 ,,	90 ,,	Three larvæ lived until the 47th day, but failed to pupate.
E.L. 1 ...	2	49 ,,	49 ,,	49 ,,	93 ,,	One larva lived until the 66th day, but failed to pupate.
E.L. 2 ...	1	18 ,,	...	...	...	On the 46th day this item of the experiment was terminated by an accident. There were 10 or 11 larvæ living at the time.

It is doubtful if the difference in mortality between the pair P.R. 1 and 2 is of any significance, but the high death rate among the larvæ given leaves is almost certainly the result of insufficiency of food.

The advantage in speed of growth between the pairs during the early days of the experiment, so far as the P.R. and C.W.L. are concerned, was clearly with the larvæ in the beakers where there had been two days for the food to soak and diffuse, or for micro-organisms to develop. With E.L. the difference was slight, which in view of the sparse growth of bacteria in the water in beaker E.L. 1 after 46 hours, is significant of some relation between the growth of bacteria and the assimilability of the food.

That the final advantage tended to be with the lots in which bacterial growth was not given any start in advance of the larvæ appears to suggest that, if bacteria and other micro-organisms are a direct source of food, they abstract a certain amount of nutriment, which is not subsequently recovered when the larvæ ingest them.

Experiment No. XXVII.

PRESENCE OF BACTERIA CONSIDERED ALSO.

This experiment was planned on similar lines to No. XXVI., but was varied as regards the description of food and number of larvæ used, in the hope of getting more nearly into touch with the problem of the relationship between larval growth and the development of bacteria in the water. An addendum, of which a short account is appended, was undertaken to allow of larvæ and pupæ being dissected to make gut smears, without interference with the breeding averages and mortality percentages in the main experiment.

Into six beakers were placed the following:—

P.R. 1 and 2, each 0·5 gr. polished rice.

C.W.L. 1 and 2, each 0·5 gr. fragments of leaves of cottonwood tree.

C. 1 and 2, each 0·5 gr. fragments of dead cockroach.

All these food materials had been thoroughly dried in a desiccator and coarsely powdered before weighing.

200 cc. of tap water was at once added to P.R. 1, C.W.L. 1, and C. 1.

All six beakers were covered with thin cotton to exclude dust, etc.

The temperature in the Laboratory at 9 a.m. during the course of the experiment averaged 82·2° F.

After three days a microscopic examination of the water for germs was made:—

P.R. 1, showed bacteria + + and a few slowly moving rounded cells (probably yeasts).

C.W.L. 1, showed bacteria + + +.

C. 1, showed bacteria + + + + +.

200 cc. of tap water was then added to P.R. 2, C.W.L. 2, and C. 2, and 10* newly hatched larvæ were placed in each of the six beakers.

1st day:—No moults.

2nd day:—P.R. 1, all but one or two are now in their second skin.
P.R. 2, many are in their third skin; the remainder in their second skin, but further advanced than any of the P.R. 1.
C.W.L. 1, mostly in their second skin; one at least is in the third skin.
C.W.L. 2, none past the second skin; all are distinctly behind C.W.L. 1.
C. 1, only two or three living larvæ to be seen; these have just attained the second skin.
C. 2, all dead (probably due to the formation of a thick, greasy scum).

3rd day:—P.R. 1, none past the third skin.
P.R. 2, a number are in the fourth skin.
C.W.L. 1 and 2, do not differ noticeably from each other; both lots are behind P.R. 2.
C. 1, only one larva now living.

5th day:—P.R. 1, is still a long way behind P.R. 2.
P.R. 2, mostly full grown, but none have pupated yet.
C.W.L. 1 and 2, mostly full grown, but no pupæ yet.
C. 1, all the larvæ are now dead.

6th day:—P.R. 1, no pupæ yet.
P.R. 2, four have pupated.
C.W.L. 1 and 2, no pupæ yet, but the larvæ are casting the chitinous tube which contains the food within the gut.

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*The eggs from which these larvæ hatched had been kept dry for three weeks; the larvæ emerged from them within one hour of immersion.

FINAL RESULT.

Lot.	Number of adults reared.	Time occupied from hatching to adult stage.			Mortality.	Remarks.
		Shortest.	Average.	Longest.		
P.R. 1 ...	5	10 days	12 days	15 days	50 per cent.	1 larva lived 34 days with abundance of food and then died.
P.R. 2 ...	4	8 ,,	9 ,,	11 ,,	60 ,, ,,	...
C.W.L. 1	9	9 ,,	11 ,,	14 ,,	10 ,, ,,	...
C.W.L. 2	6	9 ,,	10 ,,	11 ,,	40 ,, ,,	...

A comparison of these results with those obtained in Experiment No. XXVI. in respect of the larvæ fed on rice (P.R. 1 and 2), and those fed on leaves of the cottonwood tree (C.W.L. 1 and 2), brings out clearly the effect of scarcity of food on the one hand, and the possibility of water, overloaded with organic matter, being unhealthy if the larvæ are not numerous enough to cope with it, on the other.

In this experiment, as in No. XXVI., the advantage at the beginning between the pairs was again with the larvæ placed in the beakers where bacterial growth had the start; the final reverse of position between the pairs, though less marked, is still sufficiently in agreement to suggest that this is a real divergence and not a mere chance occurrence.

In the case of the pairs fed on cockroach (C. 1 and 2) the oily nature of the food material must be considered as chiefly, though not entirely, responsible for the ensuing mortality. For, even when the surface scum was broken up and a quantity of fresh water added, and these beakers produced some healthy adults, still the mortality was high.

Fifty larvæ which had emerged from their eggs an hour or two previously were added to beaker C 2, the covering of scum being broken up. They grew very slowly at first and there was a heavy death rate, but by the third day the survivors were making extraordinarily rapid progress and by the fourth day were in the fourth skin. All but one had pupated by the sixth day. Twenty-two adults were reared, eight within seven days; the average time being eight-and-a-half days, and the longest period between egg and adult eleven days.

A smear of the gut of a larva taken from this batch showed only two or three bacteria to a field, and one from a pupal gut was free so far as the microscopic test went; yet the water in which these larvæ were living swarmed with bacteria.

During the progress of Experiments Nos. XXVI. and XXVII. it became evident that the introduction of larvæ may have a remarkable clearing action on foul, cloudy water, if the conditions are properly balanced in respect of the number of larvæ and the quantity of water.

A test was made of the water from beaker C. 1. after the death of the last larva on the third day (the eighth day of the growth of the bacteria), the water showed an enormous bacterial infection, only comparable with a broth culture. This water was put into a glass dish and diluted with four times its own volume of tap water, which rendered it less dense, but left it still cloudy; two hundred first skin larvæ were then placed in the dish, they grew with great rapidity, and as they progressed the water cleared, finally entirely losing all trace of its cloudy appearance.

The water in beaker C. 2 retained its thick, cloudy appearance *after* the development of the fifty larvæ above alluded to. It was decided to use this water for a further test regarding the clearing action of the larvæ.

A microscopic examination by wet films and stained smear (carbol thionin blue) showed the water to be swarming with bacteria—three forms predominating. One of these was a large and a very distinctive serpentine form. About one hundred first skin larvæ were added to the 200 cc. of foul water; they made very rapid progress. Two days later most of them were half grown, while the water had become quite clear. Microscopic examination showed a very marked reduction in the number of bacteria, with an entire absence of the large serpentine form.

ADDENDUM.

Two beakers, in every respect similar to those used in the above experiment for P.R. 1 and C.W.L. 1, were prepared and stocked with the same number of larvæ.

Larvæ and pupæ were taken from these beakers at intervals and dissected, smears of the gut and its contents being fixed and stained in the same manner as sample drops of water. While the water in these beakers showed an identical infection with that in P.R. 1 and C.W.L. 1, in above experiment, the smears of the larval guts showed few, if any, bacteria to a field, and the pupal none at all.

Experiment No. XXVIII.

POSSIBLE RELATIONSHIP BETWEEN THE DEVELOPMENT OF BACTERIA AND THE GROWTH OF THE LARVÆ.

This experiment was a further attempt to solve the question of a possible relationship between the development of bacteria or other small organisms and the larvæ. The quantities of the different foods used were varied in the light of past experience in previous experiments.

On the 20th May, into four beakers, carefully cleaned and covered with thin cotton as in the previous experiments, were placed the following:—

Into G. 1 and G. 2 0·15 gr. of desiccated fragments of grasshoppers was placed.

Into C.W.L. 1 and C.W.L. 2, 0·5 gr. of desiccated fragments of fallen cottonwood leaves.

200 cc. of tap water was then added to G. 1 and C.W.L. 1.

On the 24th May, four days later, an examination of the water in G. 1 showed that it was thick and cloudy, while bacteria were present in immense numbers, three or four forms being especially noticeable:—

(1) A small, very short rod-shaped bacillus in dense clusters.

(2) Two forms (possibly of the same species) of large rods generally distributed but occasionally forming chains.

(3) Large serpentine forms scattered throughout the mass.

No paramecium or rotifers seen.

C.W.L. 1, water clear but with fluffy patches. Bacteria less numerous, but still in great numbers. The large serpentine forms not present. A few paramecium and a single specimen of a rotifer seen.

200 cc. of tap water was then added to G. 2 and C.W.L. 2.

16 newly hatched larvæ were placed in C.W.L. 1 and 15 in C.W.L. 2.
25 ,, ,, ,, ,, ,, ,, G. 1 and G. 2.

1st day (24 hours from start):—

G. 1, a number have moulted.
G. 2, none have moulted.
C.W.L. 1 and 2, none have moulted.

2nd day:—G. 1, nearly all in third skin and mostly well grown in it; one seen in first and one in second skin.

G. 2, a few (possibly one-third) in third skin, but the larger number in the second and two or three seen in the first skin.

C.W.L. There is not much to choose between C.W.L. 1 and C.W.L. 2; C.W.L. 1 has a few "stars" whereas C.W.L. 2 are more even in growth. Only one-fourth to one-third are in the third skin and these have only just attained it; on the other hand, there are no second skin "laggards."

3rd day:—G. 1, 1 still in second and 1 in third skin; the remainder are in their fourth skin, mostly full grown, but none have yet cleared the gut in preparation for the pupal stage. The water is now less cloudy than in G. 2.

G. 2, are very noticeably behind and have a higher death-rate. While most of them are in their fourth skin, and a few are as forward as G. 1, there are several that have only recently attained the third skin, and at least two are still in the second. Water very cloudy.

C.W.L. 1, about half are in their fourth skin and fairly grown, but slender (fat body ill developed); remainder in third skin. Water clear, brighter, and more deeply coloured than C.W.L. 2.

C.W.L. 2, are possibly a shade more backward than C.W.L. 1, but the difference is very slight.

EXAMINATION OF WATER.

4th day:—G. 1, after eight days, larvæ having been present for half the period, the water is nearly clear. Bacteria, though still plentiful, are in greatly reduced numbers. The serpentine forms and large rods are scarce, and there is also a marked absence of the dense clusters of the smaller bacilli.

G. 2, after four days, during which larvæ have been present all the time, the water is thick and cloudy, and there has been a considerable mortality. Bacteria are present in great numbers, but the large rod and serpentine forms are less numerous than they were in G. 1 before the larvæ were added. The most noticeable difference now is the absence in G. 1 of the dense clumps of short rod forms and their presence in G. 2.

C.W.L., the water in C.W.L. 1 is slightly darker than in C.W.L. 2, but both are clear and bright. There is, if very careful comparison is made, a trace of dulness in C.W.L. 1.

C.W.L. 1, shows very few bacteria, but much fluff, possibly cellulose fibre.

C.W.L., 2, shows a heavy infection compared with C.W.L. 1, mostly small forms with a few of the large chain-forming rods. No clusters and no serpentine forms.

DEVELOPMENT OF LARVÆ.

G. 1, 1 pupa, 1 in second skin, 1 in third skin; remainder full fed (fat bodies developed), 22 living.

G. 2, no pupæ; no "laggards" (probably dead), only 13 living, all of which are full-grown.

C.W.L. 1, all in fourth skin, mostly full fed, 16 living.

C.W.L. 2, all in fourth skin, mostly full fed, 14 living.

5th day :—G. 1, 17 pupæ, 3 full fed larvæ, 1 just into third skin.

G. 2, 9 pupæ, 4 full fed larvæ.

C.W.L. 1, 4 pupæ, 10 larvæ, full fed.

C.W.L. 2, 4 pupæ, 14 larvæ, full fed.

FINAL RESULT.

Lot.	Number of adults reared.	Time occupied from hatching to adult stage.			Mortality.
		Shortest.	Average.	Longest.	
G. 1 ...	15 males, 7 females	6 days (10 males, 2 females)	6·5 days	8 days (2 females)	12 per cent.
G. 2 ...	11 males, 2 females	6 days (2 males)	7·1 ,,	8 days (3 females)	48 ,,
C.W.L. 1	10 males, 6 females	7 days (6 males, 1 female)	9·6 ,,	18 days (2 females)	Nil.
C.W.L. 2	5 males 9 females	8 days (1 male, 3 females)	14·8 ,,	24 days (1 female)	7 per cent.

After the emergence of the adults in beakers G. 1 and G. 2, on the 6th day after the introduction of the newly hatched larvæ, an examination of the water was made.

G. 1 appeared nearly clear; quite clear in comparison with G. 2. The bacteria present, though numerous, were relatively few in comparison with the conditions at the beginning of the experiment. Only occasional serpentine forms and single individuals of the large chain-forming rods were to be met with; very few clusters of the small short rods and such clusters when seen were small.

G. 2 was still swarming with large clusters of the short rods, while chains of the large rods and serpentine forms were numerous; the water being very thick and cloudy.

SEQUEL.

A fresh batch of 20 newly hatched larvæ were added to each beaker.

1st day (24 hours later):—G. 1, nearly all in second skin.
G. 2, all are in their second skin.

2nd day:—G. 1, mostly just into third skin, two or three in second and one or two in first.
G. 2, are all in third skin and mostly well grown in it; they are well ahead of G. 1 and the water is now nearly clear.

3rd day:—G. 1, mostly in third skin; two still in second and one in fourth skin.
G. 2, One in third, remainder in fourth skin—nearly all full fed.

4th day:—G. 1 and 2, compared, the water in G. 2 is yellower, but both are equally clear; as regards the development of the larvæ there is no comparison.
G. 1, are marking time in the third skin; only one has reached the fourth, and two remain very small in second or third skin.
G. 2, on the other hand, are all in the fourth skin: most of them with well developed fat bodies.

5th day:—G. 1, still marking time.
G. 2, 8 pupæ; remainder full grown.

FINAL RESULT OF SEQUEL.

Lot.	Number of adults reared.	Time occupied from hatching to adult stage			Mortality.
		Shortest.	Average.	Longest.	
G. 1 ...	1 female	32 days	...	...	95 per cent. 1 larva lived until the 59th day and then died.
G. 2 ...	7 males, 9 females	6 days (2 males)	8 days	12 days (1 female)	20 per cent.

In view of the comments on Experiment No. XXVII. these last results scarcely call for any remarks; the experiment satisfactorily disposes of any remaining doubts as to the interaction of bacterial development and larval growth. The tentative suggestions put forward in the comments on Experiment No. IV., to the effect that bacteria took nutriment from the water which, even in the event of the larvæ devouring them, could not be regained, is confirmed by the results following the re-stocking of beakers G. 1 and 2 after the first broods had been reared. The success attending the careful adjustment of food to the quantity of water and number of larvæ also testifies to the correctness of the conclusions concerning these points in the comments at the close of the various experiments.

There seems little doubt but that the bacteria* themselves form a readily assimilable form of food for the larvæ, although, from the point of rigid economy, a certain wastage occurs unless the several factors are very nicely adjusted. This, however, in the terms of the case is likely to happen under natural conditions.

Experiment No. XXIX.

ABILITY OF THE LARVÆ AND PUPÆ OF *STEGOMYIA FASCIATA* TO SURVIVE COMPLETE SUBMERGENCE.

183 larvæ and 40 pupæ were placed in a wire gauze tube, 5 inches by 2 inches. The tube was then submerged in a large pan of water, the top of the tube being 2 inches beneath the surface. Some of the larvæ escaped through the 18 × 18 mesh of which the tube was constructed, and pupæ were also found outside, but these individuals may have escaped while yet larvæ.

After 20 hours an examination showed that 36 per cent. had escaped through the mesh. Of those which remained, 78 per cent. had been killed.

Pupæ— 3 living, 36 dead; mortality, 92 per cent.
Larvæ—28 „ 76 „ „ 73 „ „

No attempt was made to resuscitate the apparently dead.

Experiment No. XXX.

TEMPERATURE EFFECTS ON LARVÆ AND PUPÆ OF *STEGOMYIA FASCIATA.*

Heat.—Experiments were conducted in a tin pan about 6 inches in depth, holding 6,000 cc. of water with a surface area of 80 square inches, placed in the open on bare earth and exposed to the full heat of the midday sun during the latter half of November. These showed that both larvæ and pupæ could easily withstand such conditions, no mortality at all being observed. The test was commenced when the larvæ, from eggs laid in the pan by wild females, were half grown (after the second moult).

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* Experiments which are now being performed in collaboration with Dr. E. E. Atkin prove that washed bacteria alone afford favourable food for the larvæ, and further suggest, though this is not yet definitely established, that the presence of living bacteria are essential to the development of the larvæ.

The temperature of the water taken on three successive days was as follows, care being taken that the thermometer was placed in the shade of the side of the tin:—

24th November,	3 p.m.	... ...	101° F.
25th ,,	3 ,,	... ...	103 ,,
26th ,,	9 a.m.	... ...	75 ,,
26th ,,	3 p.m.	... ...	101 ,,

Larvæ and pupæ were taken from this pan with a quantity of the water and put into a beaker; this was placed in a water bath and the temperature slowly raised. After 10 minutes the temperature reached 110° F., the pupæ remaining active, but they all kept close to the surface. Between 112° and 115° F., reached within 15 minutes, both larvæ and pupæ died.

Larvæ from the pan were treated similarly, but the course of the experiment was interrupted; the moment the thermometer registered 112° F. the beaker was removed from the bath and allowed to cool. All the larvæ seemed to be in difficulties; most of them remained at the surface, but a few continued to show slight activity at the bottom. 20 hours later, the water having fallen to the laboratory temperature of 80° F., about one-third to one-half of the larvæ had recovered their activity or had pupated, the remainder were either dead or dying. The survivors nourished upon the dead bodies of their comrades successfully completed their development, producing adults which, so far as outward appearance was concerned, were normal.

From this it would appear that the upward limit of temperature is in close agreement with that for other insects, as shown by Blacklock (1912) with bugs and Bacot (1914) with bugs, fleas and cockroaches.*

Cold.—A number of larvæ and pupæ were taken from various breeding pans in which the water temperature was in the neighbourhood of 80° F.

The beaker to which they had been transferred was placed in the ice chest and cooled to about 74° F.; this change did not affect the insects at all. It was then placed on a block of ice; after one hour the temperature of the water had fallen to 49°–50° F. All the pupæ remained active, and one adult (a female) had emerged; about half the larvæ, including both small and large, had fallen to the bottom stiff and immovable, to all appearance dead. After a further two hours on the ice the temperature had fallen to 40° F., and all the larvæ were in stiff and stretched out positions at the bottom; while all the pupæ, with one or two exceptions, were immobile and lax at the surface. The beaker was removed from the ice and the temperature allowed to gradually rise. At 60° F. all the pupæ had regained their full activity, but the larvæ showed no signs of recovery until the temperature rose to 70° F. An hour later, when the temperature had reached 80° F., nearly all the larvæ had recovered their activity.

Next day.—6 larvæ and 2 pupæ were dead out of 40 larvæ and 24 pupæ submitted to the test; 6 adults had emerged from pupæ; from the remaining larvæ and pupæ 47 adults were reared.

The female which emerged from the pupæ while the beaker was on ice was placed in a box and put on the ice beneath the blanket. After two hours she showed no effect from the cold beyond being slightly sluggish. After twenty-four hours she was still living and active. The melting of the ice prevented a continuance of the test.

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* Adults of *S. fasciata* are quickly killed at a temperature of 50° C.

Experiment No. XXXI.

SURVIVAL OF LARVÆ OF *STEGOMYIA FASCIATA* ON WET FILTER PAPER.

A larva in its fourth skin was stranded on a piece of filter paper; after one hour's drying the paper had become stiff and dry. The larva was shrunken in appearance and incapable of movement. The small piece of paper on which it lay was snipped off and returned to the water. The larva lived and remained active for 44 hours, during which period it could not get to the surface for air, owing to one of its gills being stuck to the paper.

Two pupæ and two larvæ were found stranded on wet mud at the bottom of a half cocoanut shell. They were removed to wet filter paper and placed in a moist cell. Within twenty-four hours both the pupæ had developed and adults emerged, but failed to finally free themselves from the pupal envelope. One larva died after three days, the other lived for ten days, during which period, although kept wet, it was unable to move

Experiment No. XXXII.

ENEMIES IN THE LARVAL STAGE.

MacGregor, in his notes with reference to the *breeding of *Stegomyia fasciata* in London, states that the young larvæ are consumed by the larger ones. The high rate of mortality among newly hatched larvæ placed in breeding pans which were already stocked, or had been previously used for breeding, was noticed early in the course of the results at Freetown, but the cause was not determined. Cannibalism among larvæ of the same age, though watched for under starvation conditions, was never detected. What appeared to be a flagrant case proved, on close observation with the binocular dissecting microscope, to be two fully grown larvæ, one half-grown and a very small one, with their jaws hopelessly entangled in a small mass of silk or hairs, probably a fragment of some insect cocoon. To the unaided eye the presence of the smallest larva was not apparent, and appearances suggested that two full-sized larvæ were attacking a smaller one. After watching half-an-hour's vain struggles, a few moments' aid with dissecting needles set all four free.

Larvæ and pupæ which died in breeding jars and pans where food had been purposely cut down to a minimum were noted as being untouched until the process of decay set in, when they soon disappeared, and a spurt by one or more of the survivors suggested that the nutriment set free had been absorbed, even if the carcase had not been eaten piecemeal.

Experiments carried out in London after my return home confirm MacGregor's observation. Newly hatched larvæ when about to moult are apparently devoured by those which have attained their fourth instar, but it is questionable if danger results from the presence of third instar larvæ. A single third instar larva was placed in a pan with 9 newly hatched ones, after three days, no food being present, 8 living and 1 dead larvæ were present in the pan, in addition to the third instar larva.

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* "Journal of Tropical Medicine and Hygiene," Sept. 1st, 1915; No. 17, Vol. XVIII., pp. 193-6.

Result, negative.

6 newly hatched larvæ were placed in a glass pan with 5 larvæ in their fourth skin. After 24 hours all the young larvæ were present, some of them having moulted.

23 more newly hatched larvæ were added to the pan and 7 more fourth skin larvæ, so that the numbers stood at 12 fourth skin and 29 young larvæ, mostly newly hatched.

After 48 hours all the young larvæ were still living and one of the fourth skin larvæ had pupated.

After 72 hours the young larvæ were still living.

After 96 hours the young larvæ were still living, and were many of them in their fourth skin, while several of the fourth skin larvæ had pupated.

Result, negative.

A fresh trial was made with—

51 newly-hatched larvæ and 20 fourth skin larvæ, in various stages of growth. The pan was of the same size as in the previous experiment. After 20 hours only 35 of the newly-hatched larvæ could be found, 31 living and 4 dead—a mortality of 31 per cent. = a ratio of four-fifths to each fourth skin larva. Of the living larvæ 13 had attained the second skin.

Result, positive.

40 larvæ that had completed their first moult were then placed in the pan with 22 larvæ in their fourth skin.

After 48 hours all the second skin larvæ were still living, a number having moulted for the second time.

Result, negative.

12 newly hatched larvæ were placed with 5 in their fourth skin into a small glass dish.

After 48 hours there were only 10 of the small larvæ present and 1 of these was dead, a loss of 16 per cent. = a ratio of two-fifths to each fourth skin larva.

Result, positive.

Test in filtered Manure Water.

200 newly-hatched larvæ were placed with 20 fourth skin larvæ in various stages of growth.

After 24 hours only 176 of the young larvæ could be found, a loss of 12 per cent. = a ratio of $1\frac{1}{5}$ newly-hatched to each fourth skin larva.

Result, positive.

88 newly-hatched larvæ were placed with 4 in their fourth skin.

After 24 hours only 82 of the young larvæ could be found, a loss of 7 per cent. = a ratio of $1\frac{1}{2}$ to each fourth skin larva.

Result, positive.

70 newly-hatched larvæ were placed with 10 in their fourth skin.
After 24 hours only 68 of the young larvæ could be found, a loss of 3 per cent. = a ratio or $\frac{1}{5}$ to each fourth skin larva.
Result, positive.

Test in clean Tap Water.

110 newly-hatched larvæ were placed with 16 in their fourth skin.
After 18 hours only 106 of the young larvæ could be found, a loss of 4 per cent. = a ratio of $\frac{1}{4}$ to each fourth skin larva.
Result, positive.

179 larvæ in their first skins, most of which had just emerged from the egg, were placed with 20 in their fourth skins.
After 24 hours all the young larvæ were present but 3 were dead.
Result, negative.

332 newly-hatched larvæ were placed with 14 in their fourth skins.
After 18 hours only 316 of the young larvæ could be found, a loss of 5 per cent. = a ratio of $1\frac{1}{7}$ to each fourth skin larva.
Result, positive.

A number of dissections were made of the fourth skin larvæ used in the above tests and also of fourth skin larvæ in various stages of growth which had been kept for from two or three hours up to a day or longer in jars of water with large numbers of newly-hatched ones. No remains of small larvæ, however, were traced in either stomach or intestine. Close observation on several occasions revealed no cases of ingestion and no desire or attempt was ever evinced by large larvæ to catch the small ones. Even under such crowded conditions that accidental ingestion seemed inevitable the act was never witnessed. The sole direct evidence of ingestion obtained was the head capsule of a first skin larva recovered from fæces passed by a fourth skin larva. The conditions under which this was found, however, preclude it being a case of the swallowing of a cast skin unless it had remained in the gut for two or more days, as none of the young larvæ had moulted during the period of the experiment.

On the whole the evidence (especially the frequent finding of *dead* first-skin larvæ in containers in which fourth skin larvæ are present) seems to point to the devouring of the young larvæ by the larger ones being a matter of chance. There remains, however, the possibility that cannibalism is a variable habit occurring in a few larvæ or certain strains of the insect only.

It seems therefore that the fourth instar larvæ may be themselves among the most formidable agents in the destruction of their species. The habit of breeding in small collections of water, while saving the species from a death toll by other enemies, enhances the opportunities and danger of fratricide. The action of the two habits, together with the intermittent hatching of the eggs, will be that of a sliding scale to keep the adult population at a comparatively even level. It will be a matter of some interest to see if the same habit of fratricide obtains in other species.

Apart from either competition or fratricide, breeding in small scattered collections of water largely precludes the action of predatory enemies during the larval stage of this species. Ducks, if free to roam about the precincts of the dwelling, will doubtless do an enormous amount of useful work ; they will, however, destroy other predatory enemies of mosquitoes, such as frogs, dragon fly larvæ and water beetles, which may be present in the larger pools, without discrimination. This does not seriously militate against their services, however, as most of the larger predatory insects, at any rate, are likely to have a wider range than mosquitoes, and will tend to rapidly repopulate from outside the areas cleared by ducks.

Fish, which are of great value in holding in check species of mosquitoes which breed in permanent pools or streams of water, are likely to be of small value as enemies of *S. fasciata*, although species with similar habits to those of carp and tench might be utilised in water butts or cisterns.

Tadpoles, as stated by Stephens and Christophers (1908) and other authors, do not attack mosquito larvæ, though they probably have a checking influence owing to competition in the matter of food supply. Direct experiment proved that even in a crowded jar there was no mortality to well grown mosquito larvæ, transformation to the adult stage taking place as usual. It is possible, however, that the small larvæ undergoing the first moult might be engulfed.

A small water-bug belonging to the *Micronecta* was found in very small rock pools with larvæ of *S. sugens* and *Anopheles costalis*. In captivity it attacked and sucked the juices of larvæ of *S. sugens*. Its power of flight would enable it to transfer itself from pool to pool if scarcity of food or drought rendered them untenable, but the species was never seen in sufficient numbers to warrant the supposition that it would do more than take a moderate toll of the larvæ it fed on.

The presence of a species of *Ostracoda*, probably belonging to the genus *Cypris*, in breeding pans or jars appears to be inimical to mosquito larvæ. This seems to be due to competition in respect of food solely, as the larvæ (*S. fasciata*) added to water containing the *Ostracoda* live for a longer or shorter time, but seem unable to proceed with their development, few, if any, succeeding in attaining the adult stage. A similar phenomenon has been noted in England with regard to *Culex pipiens*—small pools in Epping Forest which were populated with a minute crustacean being free of mosquito larvæ.

(*C*) ADULTS.

General Notes.

Pairing and feeding with *Stegomyia fasciata in captivity* are not restricted to any particular period of the day or night. No doubt if an accurate census could be taken, the late afternoon or evening might be found to be the most favoured period, but when numbers of recently emerged specimens are placed in a cage pairing commences almost immediately, and feeding, on the part of a proportion of the females, at the earliest opportunity.

There seems to be no regular precedence of one or the other function, but females heavy with blood are too sluggish to pair and tend to rest low down in the cage, or even on its floor. Anything less than a full meal only slightly impairs their activity and pairing proceeds as usual. If the initial meal of blood be interrupted they will feed again within the next few minutes, satiety being evidently instinctively aimed at.

Feeding.—The effect of drought as a deterrent to feeding was very noticeable, both in the cages within doors and especially in the mosquito house out of doors.

In the cages, both by day and at night, the harmattan periods did not interfere with feeding and egg laying to any extent, because the time allowed for feeding was ample, but in the mosquito house, where the visits were of short duration, the effect of dry periods was much more noticeable. In place of the surging swarms which usually rushed on their prey, only a few individuals put in an appearance to feed on my entrance. Within a few minutes, however, the insects issued from their hiding places and commenced to attack. The evidence as a whole suggests that, while the dry air is the cause of retirement to any available shelter from the wind, it is not disinclination to feed that prevents the mosquitoes biting so readily, but failure to recognize the presence of a host. Probably it is the absence of odour which delays attacks in dry weather. The bites, if numerous, have the effect of inducing a profusion of perspiration, especially in warm weather and before one becomes thoroughly inured to their attacks, while the free flow of perspiration seems to attract the insects.

Pairing commences during flight, but is usually consummated while at rest, and occupies but a few moments at most. The position when the pair have settled varies considerably. A tail to tail position was observed, but is rare; side by side, with bodies tilted at an angle to each other and the surface on which they are resting is not uncommon, but the usual position seems to be for the male to embrace the female and underlie her with his back to the resting surface.

General observation suggests that the same female, possibly the same pair, copulate several times in quick succession, but no experimental proof of this was obtained when a single pair were segregated.

Captive females refuse to suck blood for some hours, in most instances for at least a day, prior to egg laying, and, if the bulk of their egg masses are not extruded on the first night, they will not as a rule feed again until after the deposition of the remaining eggs. Evidence is afforded by the record of the female used in Experiment No. IX. on page 34.

Vigorous females almost invariably feed within twenty-four hours after clearing their ovaries of mature eggs, and on the following day as well. On the third or fourth day they usually abstain, a fresh batch of eggs having developed. Weak or elderly females are much more erratic, both as to feeding and the deposition of eggs. There is direct evidence in Experiments Nos. XLI. and XLII. of the retention of eggs for considerable periods by some females. The proportion exhibiting the habit is not large, and the period of retention is variable; while it is possible that further meals of blood would have induced some of these females to deposit their eggs earlier, it is to be noted that the habit was observed in a few instances in the case of females which were afforded nightly opportunities of sucking blood. Probably the habit affords evidence of a more or less latent instinct, connected with æstivation, which preceded the evolution by the various species of *Stegomyia* of eggs so perfectly adapted to resist drought as those which are now produced.

A single full meal of human blood was found to be sufficient for the development of the ovaries. Eggs were deposited in many instances after a single meal of blood, and trial showed that these eggs were fertile.

Females fed on a goat, dogs, a ground pig (Bandicoot) and wild rats *(Mus rattus)* also developed their ovaries after a single meal of blood from these animals. In the case of those fed on *Mus rattus* trials were carried out to see if the eggs were ripe for deposition. A female was placed in a jar with wet filter paper and, after the lapse of a few days, deposited a batch of eggs which, after incubation, hatched within a few minutes of their immersion.

The laying of eggs on any diet other than circulating blood is a rare but not unrecorded phenomenon. Otto and Neumann (1905) succeeded in obtaining fertile eggs when the females had been fed on blood and salt solution. Two instances occurred in the course of the present research of single eggs being laid by females that had no opportunity of feeding on a living animal. In one case the females were fed on blood mixed with honey, in the other on blood mixed with syrup. In the latter instance the egg proved to be fertile. These foods were chosen with the special object of testing the practicability of breeding without allowing them to feed on a living animal, as the practical advantage of such a method would be very great. In the face of the extreme rarity of egg-laying under the conditions, however, the experiments must be considered as failing of their purpose.

Eggs were only deposited when water or saturated surfaces were available. An experimental test (No. XLIII.) carried out with a view to confirm this general observation resulted in a single infertile egg being laid by one of thirty-nine regularly fed and presumably fertilized females.

Length of Adult Life.

It is impossible to ascertain the length of life of adult mosquitoes under natural conditions. Even under semi-natural conditions the difficulties are great and increase just in proportion as nature is simulated.

The larger the containing cage, the greater the difficulties of counting and obtaining any exact figures of the mortality, while reduction in size leads to difficulties in feeding and the introduction of specimens.

In either case a small mesh netting (18 × 18 to the inch for *S. fasciata*) must be used to obviate escape; this greatly militates against a free draught of air, and in the case of small confining chambers it becomes only practicable to record the temperature and humidity in the immediate vicinity exterior to such cages.

Probably, while the shielding afforded by captivity tends to reduce the general mortality, no artificial conditions can be arranged so favourable to the exceptionally long-lived and lucky mosquito as those obtaining in freedom.

The ability to shift its resting place to meet the needs of every change of humidity or temperature both daily or hourly is an asset which is reduced to such narrow limits by captivity as to be all but valueless.

Two plans were followed to obtain feasible results in this research ; the one of confining 50 or 100 adults in wire gauze cylinders 4½ ins. × 2 ins., the other of keeping them in the convenient cage that can be made out of a large sized hurricane lamp chimney, the top covered with fine gauze while the open lower end is placed downwards on a pad of either dry or wet filter paper placed in a wide shallow tin lid.

The first method allowed of the nearest approach to natural conditions of drought or humidity, it was possible to devise with the object in view, while the latter afforded a more favourable way of testing the effect of different foods, egg-laying, etc. The wire gauze tubes were placed in a variety of situations but the necessity of making use of some device to prevent the ants reaching them considerably limited the possible range.

Mosquitoes were allowed to emerge direct into the tubes, a plan that avoided much waste of time and allowed of accuracy in respect of their age, but had the disadvantage that records of sex and numbers could only be accurately ascertained after their death. The sex has been recorded wherever possible, but when the insects were placed in damp situations the remains were frequently too mildewed or decayed to admit of their sex being discriminated ; while in some instances the actual numbers after the mid period of the experiment are approximate only, owing to the same causes. Such situations as the Well in the Sanitary Department Compound, the Pit in the Mosquito house, and, in some cases, the Yard at 26, Westmoreland Street, led to trouble in this respect.

Ants were responsible for the wrecking of a number of experiments and caused both waste of time and effort, while the rotting through of the cord or rope used to lower the tubes and isolation pan down the well was a source of annoyance.

In all the experimental records the period of life tends to err by the length of the period between each examination. In every case the extreme has been taken, the mosquitoes being assumed to have lived up to the date on which they were found to be dead. In most cases the examination was daily, but in the Pit it was occasionally every other day, while in the Well only two examinations a week could be made, so that the error might amount to three or four days.

It will be safe, therefore, to assume that the lives recorded are the limits under the particular conditions involved.

Without food the adult life, under favourable conditions, although short, is by no means inconsiderable for so small and active an insect, an average of 6 to 8 days, with an extreme of 12 days, being recorded. This compares favourably with the records in which food was given in less suitable situations. It would appear to be a general rule that the environmental conditions are more important than the food; honey and syrup given under moist conditions giving a longer average life than blood under dry ones. Another point which the budget of record shows is that the kind of food given makes far less difference to the average length of life than it does to that of the exceptionally long lived individuals.

The second plan was used in the attempt to ascertain the extreme age limit under favourable conditions when fed on various foods.

Hurricane lamp chimney cages were also employed in the experiments dealing with egg production on a single meal of blood and the attempt to induce egg-laying by giving a mixture of blood, either fresh or dried, with honey or syrup as food. The air in these cages would be still, if not stagnant, so that the use of a dry, in place of a wet filter paper, as a base in some of the trials, would not necessarily cause a drying atmosphere, though of course it would be dry if compared with the saturated conditions which obtained when the base rested on wetted paper and the gauze top was covered with a piece of glass. All the experiments in which these lamp chimney cages were used were carried out at the quarters in Westmoreland Street, so that the working room temperature and humidity chart applies to all of them.

In the second series the advantages of moist over drier conditions are far less apparent, in fact, the suggestion is rather that humidity may be in excess of what is desirable from the mosquito's point of view. This impression is, however, partly due to an accident causing a high concentration of the syrup given as food in one of the experiments, and also to the fact that a number of the insects got their legs into a sticky condition, and this apparently raised the death rate at an early period of the experiment. On the whole, syrup gave a better result than honey, possibly because it was more fluid. Banana, again, gave less favourable results than either honey or syrup; this may also be owing to the fluids in the over-ripe fruit being less easily obtainable than syrup from cotton-wool. The longest female lives recorded in the series are of those fed on a mixture of blood and syrup or blood and honey. The average ratio of life of the males in comparison with that of the females would seem to be from about 3 to 5 or 2 to 3.

An experiment (No. XLIII.) carried out with the double object of ascertaining if the females would lay eggs apart from water or wet surfaces, and their length of life when well fed on human blood under these conditions, shows that such circumstances are not detrimental to longevity, the average life of 31·3 days, with an extreme of 74 days, comparing not unfavourably with that recorded when they are afforded opportunities of oviposition.

Experiment No. XXXIII.

CHOICE OF SITUATION FOR OVIPOSITION BY FEMALES OF *STEGOMYIA FASCIATA*.

At an early stage of the research trials were commenced to see if ovipositing females lay their eggs in any receptacle containing water without reference to its situation or if, as is commonly stated, they show a preference for certain rooms or positions. Various utensils containing water were placed in the basement, outbuilding, yard, living room, bedroom, etc., in the quarters at 26, Westmoreland Street.

A careful search was made every three or four days for a month. The record is as follows:—

Bedroom.—1 larva, *S. fasciata.*

Bathroom.—A few larvæ and ova of *S. fasciata*, also a batch of eggs of *Culiciomyia nebulosa.*

Covered Wooden Gallery (containing pantry and sink where the "boys" wash up).—A few eggs and larvæ of *S. fasciata.*

These latter occurred in a water pan in which one of the legs of the safe was placed. It is possible that they resulted from eggs left over from the previous tenancy. The *basement*, *yard*, *kitchen*, *store-room*, *dining-room*, and *living-room* were all negative.

This preliminary trial was followed by another, covering the month of October, in which new card cream jars of one pint capacity were used, the jars being half full of filtered rain water. The situations were as follows: —

Bedroom.
Dining-room.
Sitting-room.
Store-room.
Hall.
Basement fronting on the street, kept closed but opening on the yard through an unglazed window.
Gallery with pantry and sink.
Kitchen.

Bathroom.
Room beneath bathroom where the house boy slept.
Cellar beneath kitchen.
Yard.

Careful search for eggs and larvæ was made every three or four days (twice a week).

RESULT.

Only the jar in the kitchen was visited. The water in this jar became very thick and foul owing to dust, dirt and insects falling in. Numbers of eggs of *S. fasciata* were laid in it. Although the jar in the gallery was not visited two of the water pans in which the legs of the safe stood had eggs laid in them, and a jar of water in the sitting-room in which some cut plants were placed also received the attention of an egg-laying female. The small jar placed in the yard was not visited, but a large tin bowl, put out with the object of getting a supply of rain water, was, and soon produced numbers of larvæ of *S. fasciata*, among which were some of *Eretmopodites quinquevittatus* and *S. luteocephala*.

In the mosquito house a wooden tub, several tin pans, and basins, and a new galvanized iron pail were used. Both the tins and wooden tub were favoured; the pail was avoided, and several successive lots of larvæ put in died off almost immediately.

It was not until after the pail had been twice thoroughly scrubbed out with soap and water, and allowed to stand in the sun for a week, full of rain water, that it became a possible breeding place for *S. fasciata*. Altogether, with the various tests in between the scourings, etc., it took a matter of six weeks to two months before the pail was serviceable for breeding purposes.

Experiment No. XXXIV.

BAITS TO ATTRACT PREGNANT FEMALES.

The perusal of minute Ref. C.O. 51,476, 24th December, 1914, together with the attached papers concerning a mosquito trap suggested by Dr. E. Peter, of Duala, Kameroons, led to further experiments designed to throw light on the extent to which ovipositing females select water contaminated with organic matter in preference to clean.

1st Trial.—A number of freshly emerged *S. fasciata* were put into a large cage. Honey was supplied for the males while the females were afforded ample opportunity of imbibing human blood every night.

Two beakers, each containing 200 cc. of tap water, had six slips of filter paper arranged with one end in the water and the other turned over the rim.

In one, *A*, nothing was added, in the other, *B*, two dead grasshoppers were placed. Both were placed in the cage containing the mosquitoes, and their relative positions were reversed during the course of the experiment.

After five days the water in *B* was cloudy and thick with a scum on the surface; it also smelt badly. The water in *A* was not appreciably less clear than at the start.

In *A*, 62 eggs (14 per cent. of the total) were laid on the filter paper at various distances between water level and one inch above it, mostly about midway up.

In *B*, 392 eggs (86 per cent. of the total) were laid, 28 on the surface scum, the remainder on the filter paper over a much wider area above water level than in *A*.

2nd Trial.—Into the same cage three beakers prepared as previously were placed.

Into *A*, tap water only.
Into *B*, 1 cc. of sheep's bile that had been kept for three months.
Into *C*, ·5 gr. of Bile Salts (Sodium Taurochlorate).

After two days in the cage eggs had been deposited as follows:—

A.	58 eggs on the filter paper	65 eggs = 23 per cent. of the total laid.
	7 eggs on the glass or water surface	
B.	71 eggs on the filter paper	100 eggs = 35 per cent. of the total laid.
	29 eggs on the glass or water surface	
C.	99 eggs on the filter paper	119 eggs = 42 per cent. of the total laid.
	20 eggs on the glass or water surface	

3rd Trial.—Four beakers were prepared as previously.

Into *A*, tap water only.
Into *B*, ·5 gr. of dried and powdered cockroach.
Into *C*, ·5 gr. of dried and powdered leaves of cottonwood tree.
Into *D*, ·5 gr. of dried crushed rice.

The beakers were then placed in an incubator at 95° F. for 24 hours and then put into the cage with the mosquitoes for two days, their positions being changed about after the first day.

After two days in the cage eggs had been deposited as follows:—

A.	418 eggs on the filter paper	433 eggs = 26 per cent. of the total laid.
	15 eggs on the glass or water surface	
B.	667 eggs on the filter paper	787 eggs = 48 per cent. of the total laid.
	120 eggs on the glass or water surface	
C.	160 eggs on the filter paper	188 eggs = 12 per cent. of the total laid.
	28 eggs on the glass or water surface	
D.	231 eggs on the filter paper	237 eggs = 14 per cent. of the total laid.
	6 eggs on the glass or water surface	

In all 1,645 eggs were laid.

The water in *B* had a whitish surface scum and smelt badly. In *C* it was pale brown and smelt rather like weak tea. In *D* it was thick and cloudy without any noticeable smell.

While the evidence of selection in all three trials appears definite, it is very far from being of the unanimous unvarying character that might be expected from a deep seated instinct coupled with larval needs. On the contrary, it carries with it a certain suggestion of bias on the part of the females in reference to their own tastes, largely parallel but not necessarily identical with provision for their offspring; otherwise, why the neglect of the suffusion of leaves, possibly bitter, and rice water, possibly sour, either of which would afford better chances for larval development than plain water?

Experiment No. XXXV.

LENGTH OF LIFE WHEN CONFINED IN WIRE GAUZE TUBES UNDER VARIOUS CONDITIONS.

In considering the relative length of life as recorded in the various trials, the situation as well as temperature and humidity must be borne in mind, as local conditions have a quite considerable influence on the amount of drought and circulation of the air.

Charts recording the temperature and humidity in the various places where the tests were made, follow the descriptions of these situations. In addition, a chart compiled from the official Meteorological Report of the 9 a.m. readings at the Freetown Meteorological Station is appended as a guide to the general conditions obtaining during the months that no records were taken in the various trial situations. The record for the well only covers the actual periods of the tests and is not translated into a curve.

A partial record of the maximum and minimum temperatures and weekly extremes is also appended. This covers the period from September 11th to December 4th in the laboratory, from December 4th to February 27th in the animal house, and from February 27th to July 31st in the mosquito house, and affords some idea of extremes registered in these three situations. As only one registering thermometer was available, it was a matter of choice between one complete or several partial records.

A record of temperature and humidity in the laboratory is included in the "General Notes" prefacing the larval section.

Yard at Quarters, Westmoreland Street.—This was small, overhung by trees, surrounded on three sides by buildings and on the fourth by a high wall. No weeds were present on the small plot of soil where the pen was fixed. On the whole a cool, moist, favourable situation. (*See* page 92).

Cage in Quarters, Westmoreland Street.—This stood on a table about 4 feet from the ground on the first floor facing the stairway. This situation was dark, a few gleams of late afternoon sunlight being the most that ever penetrated to the cage, but, on the other hand, it was well ventilated. (*See* page 93).

Compound at the Hospital.—The pen was placed in an open, sunny spot, slightly shaded at midday by eucalyptus trees. The insects confined here were subjected to greater extremes of heat and drought than in any of the other situations. (*See* page 94).

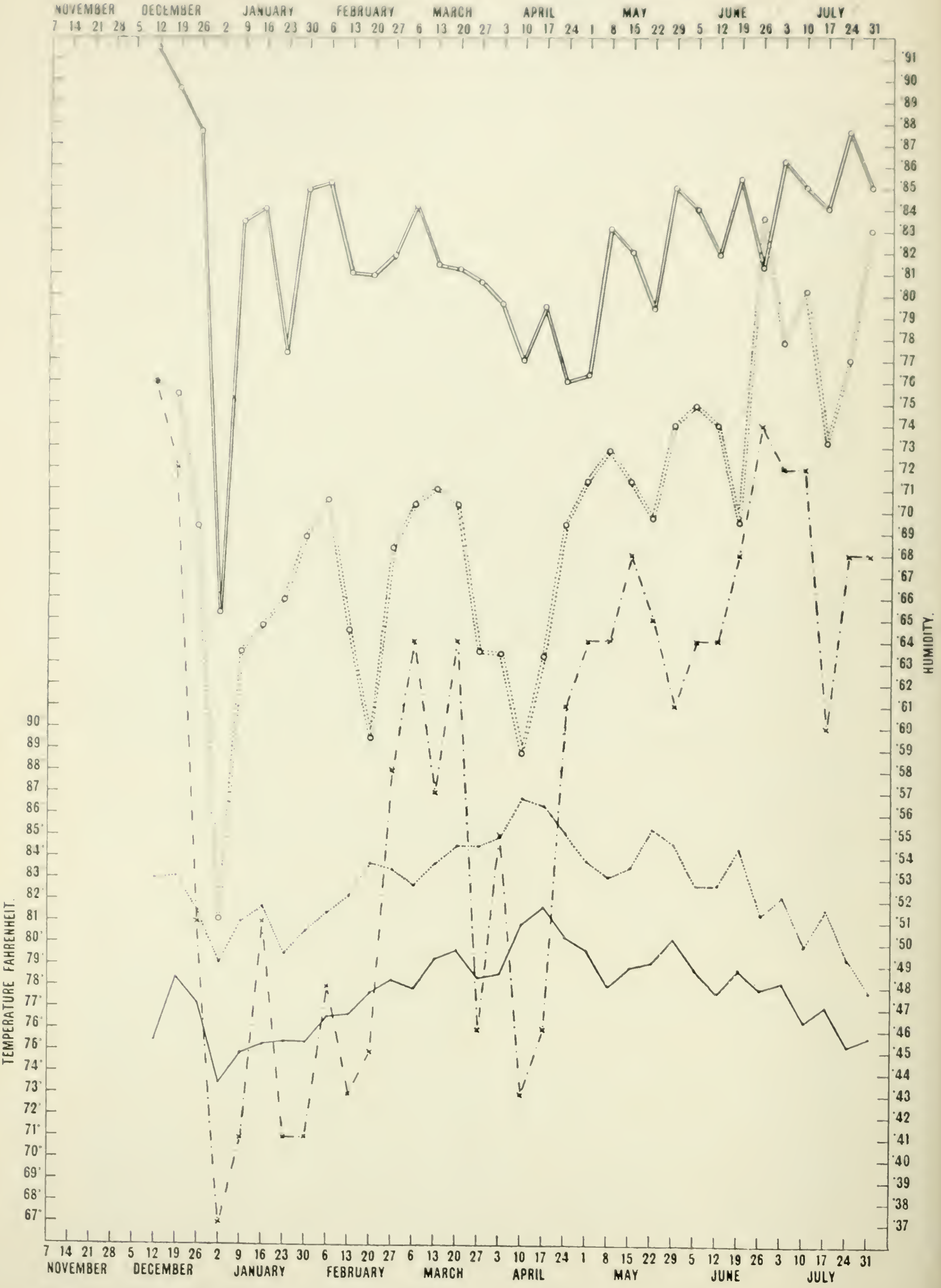
Animal House.—(Plate No. 20, page 96). Darker, cooler and less well ventilated than the cage at quarters in Westmoreland Street. The air would also be cooler and more moist at night. (*See* page 95).

Mosquito House.—(Plate No. 21, page 96). This was sheltered under a small but densely leaved tree and also by a tall shrub. It was shut in on east and south by buildings and on the west by a high wall a few yards distant from it. On the north it was exposed, but obtained some shelter from two small trees about ten yards away. On the whole, a shaded situation tending to be both cool and moist. (*See* page 97).

Mosquito House Pit.—The pit within the Mosquito House was two feet by two-and-a-half feet in area, and two feet deep, lined with rough planks. It was covered by a wooden lid, which left an open crack half an inch wide. This situation was very dark, ill-ventilated and humid. (*See* page 98).

CHART No. 2.

CURVES OF TEMPERATURE AND HUMIDITY IN YARD AT 26, WESTMORELAND STREET, FREETOWN, SIERRA LEONE.



Observations made at 7 a.m. and noon each day :—

Temperature ———————— 7 a.m. Humidity ════════

„ . . . . Noon „ : : : : : : : : : : : :

Minimum Humidity records in each week — · — · — · — ·

Chart No. 3.

Curves of Temperature and Humidity in Working Room at 26, Westmoreland Street, Freetown.

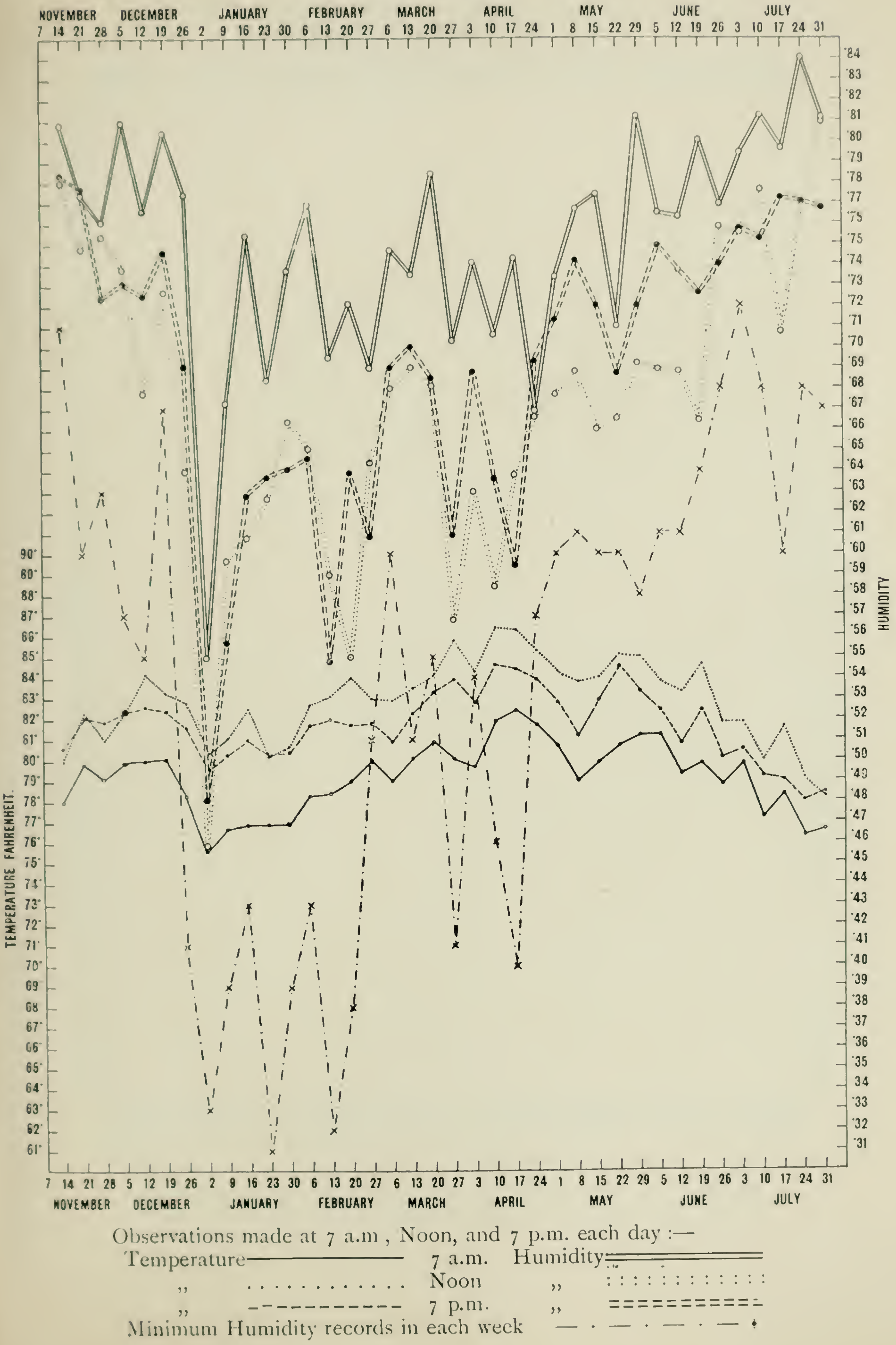
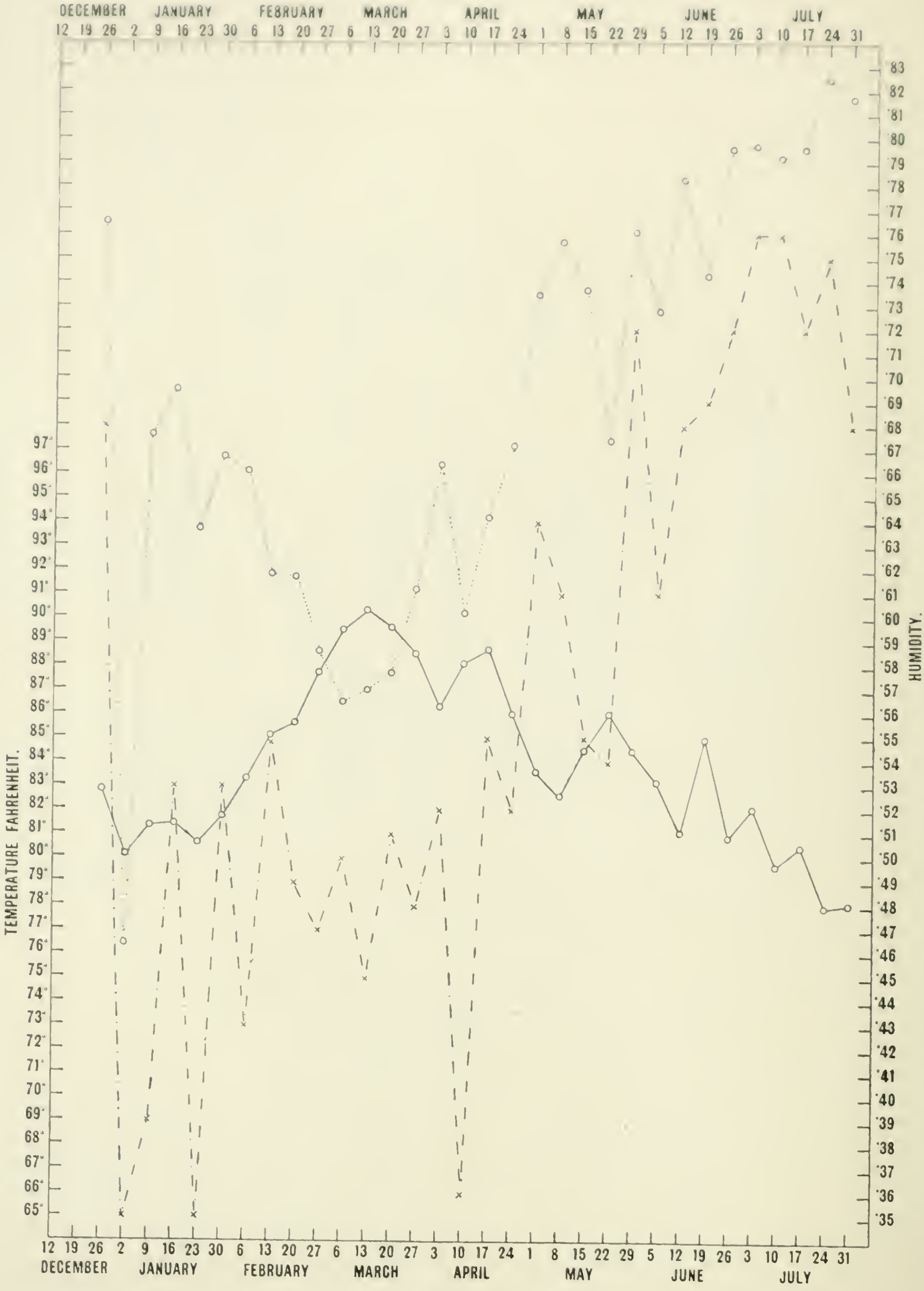


CHART No. 4.

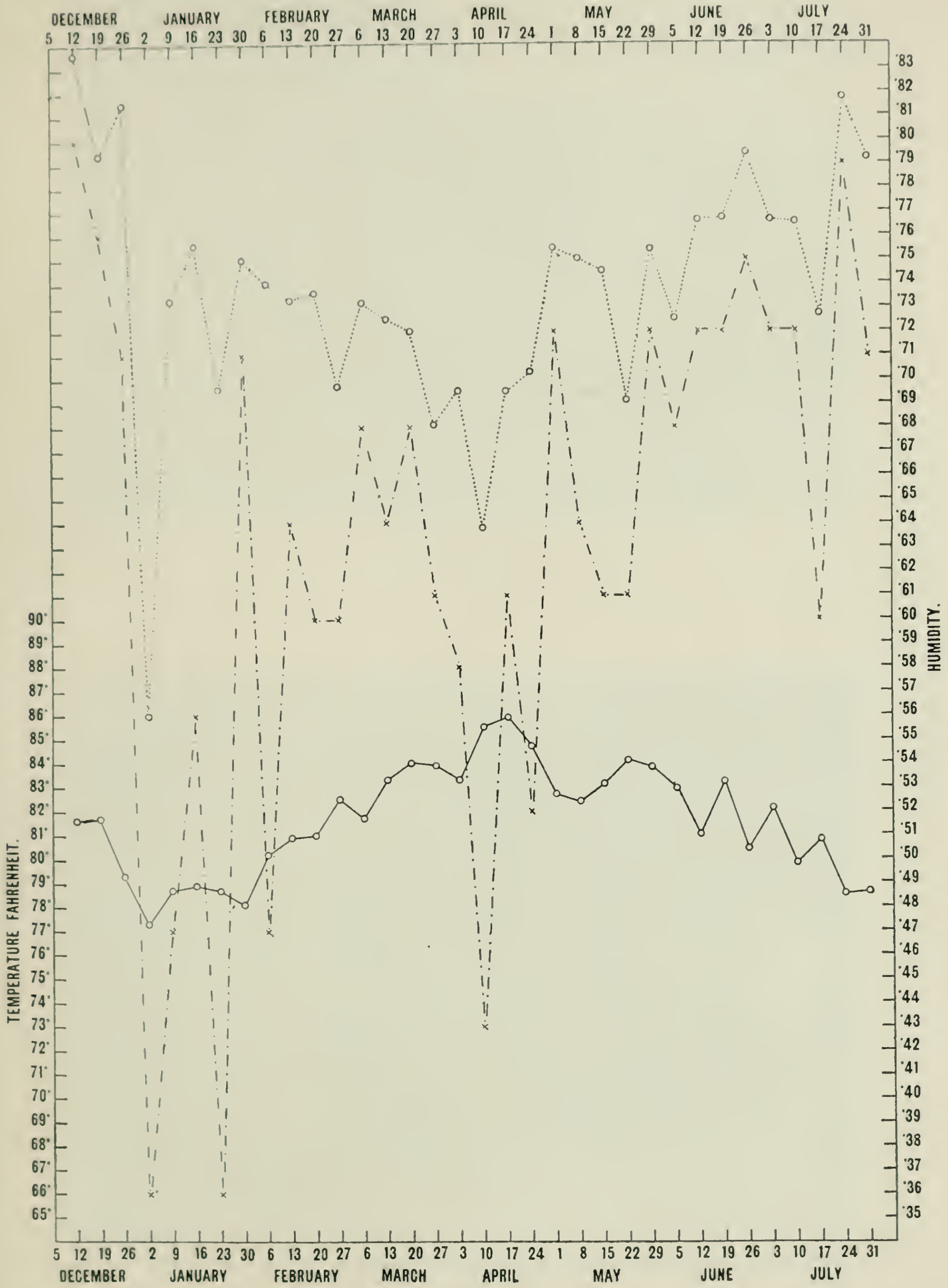
CURVES OF TEMPERATURE AND HUMIDITY IN COMPOUND AT COLONIAL HOSPITAL, FREETOWN.



Observations at 9 a.m. each day :—
Temperature ————————
Humidity . . . .
Minimum Humidity record in each week — · — · — · — ·

Chart No. 5.

Curves of Temperature and Humidity in Animal House at Colonial Hospital, Freetown.



Observations at 9 a.m. each day :—

Temperature———————— Humidity . . . .

Minimum Humidity record in each week — · — · — · — ·

Plate No. 20.

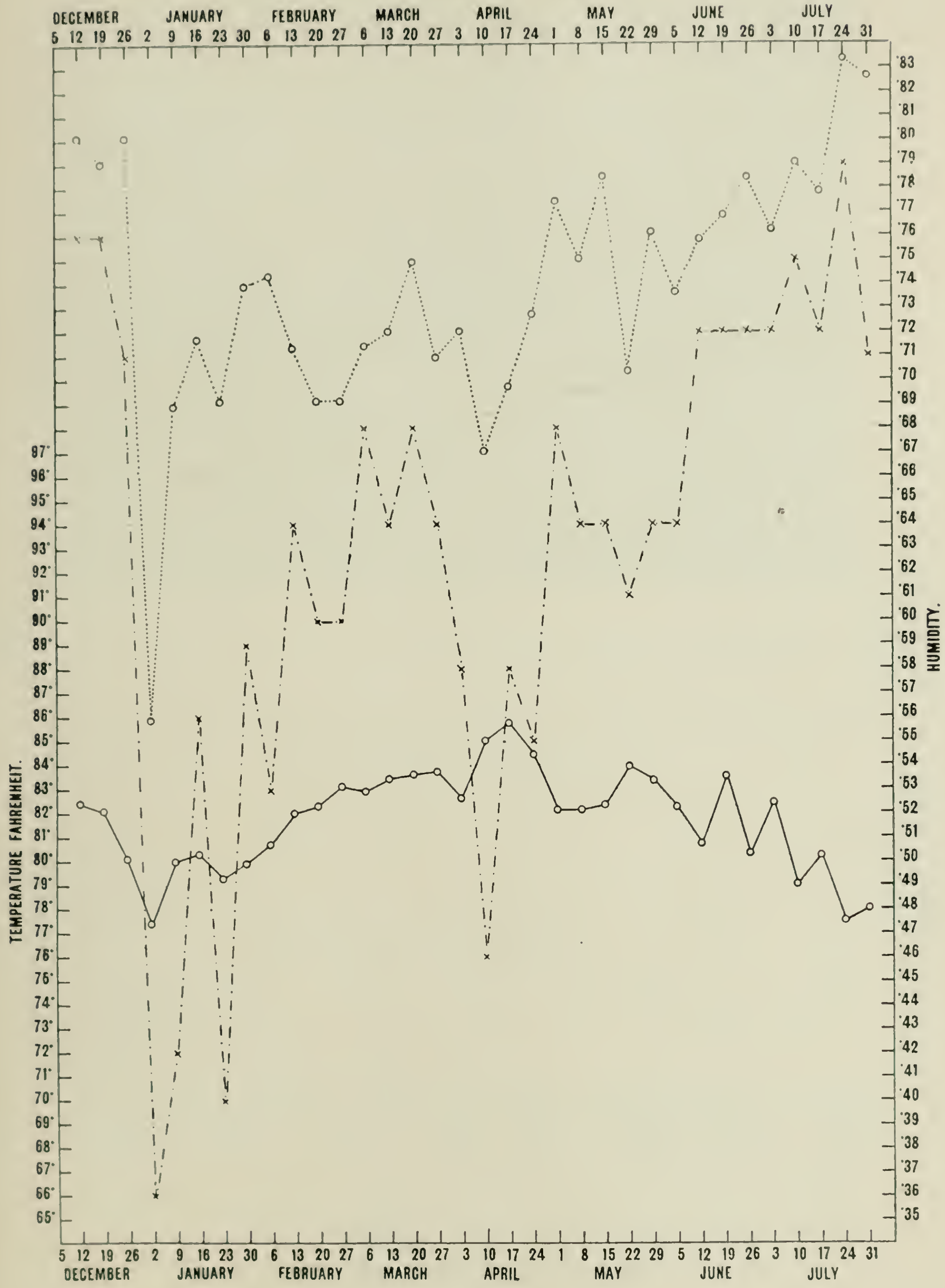
Animal House, Colonial Hospital, Freetown.

Plate No. 21.

The Mosquito House.

CHART No. 6.

CURVES OF TEMPERATURE AND HUMIDITY IN MOSQUITO HOUSE AT COLONIAL HOSPITAL, FREETOWN.

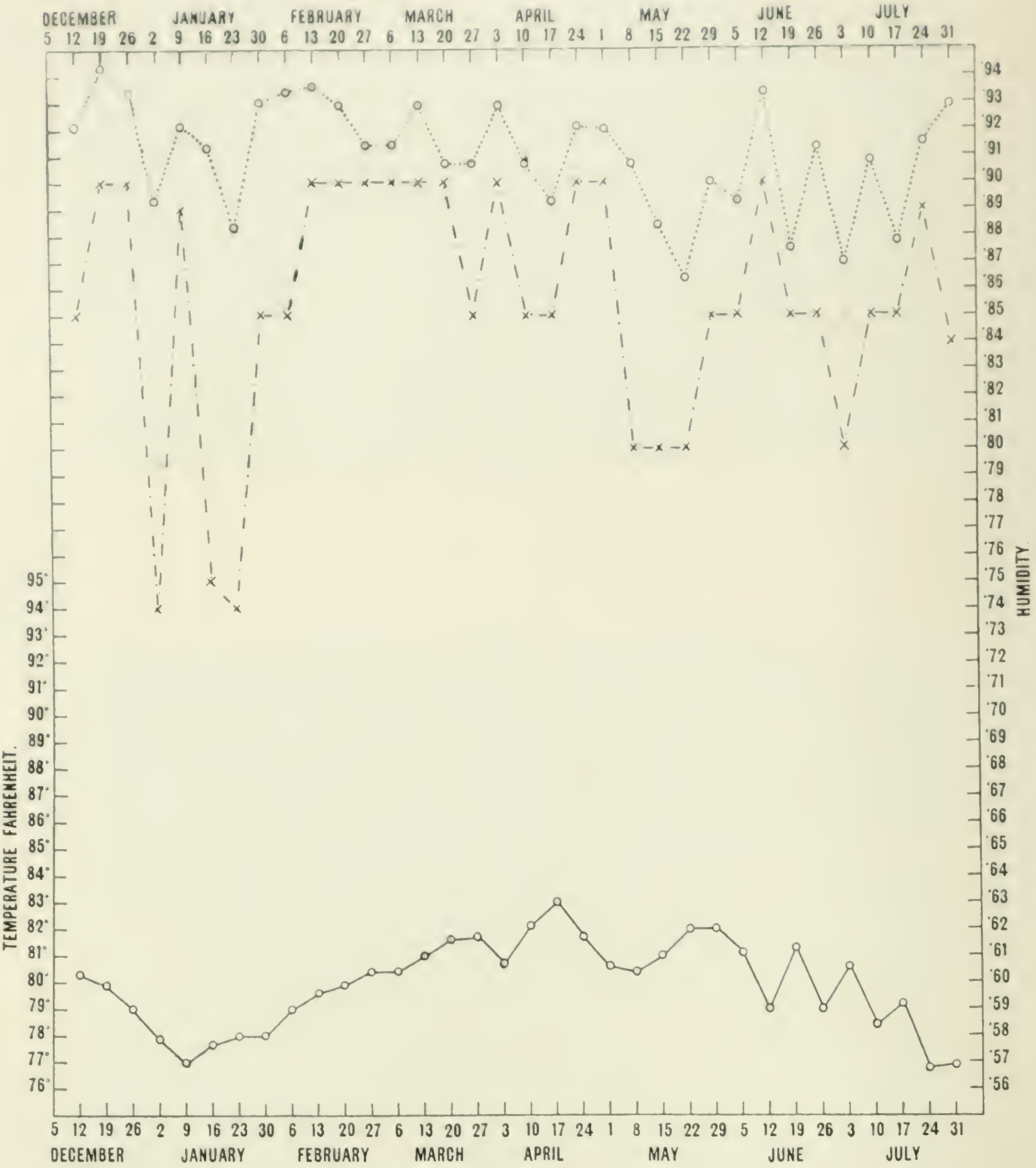


Temperature———————— Humidity . . . .

Minimum Humidity record in each week— · — · — · — ·

CHART No. 7.

CURVES OF TEMPERATURE AND HUMIDITY IN THE MOSQUITO HOUSE PIT.



Observations at 9 a.m. each day:—

Temperature——————— Humidity . . . .

Minimum Humidity record in each week— · — · — · — ·

Well.—(Plate No. 22). This was covered, but owing to the larger air space it was better ventilated and less humid than the pit in the mosquito house.

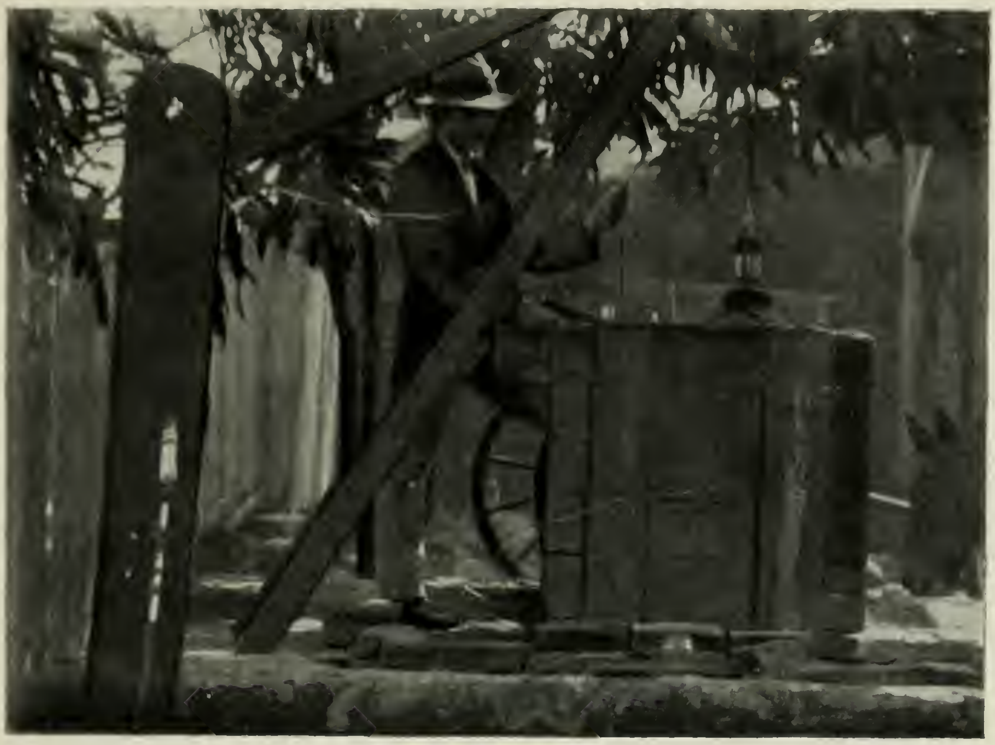


Plate No. 22.

Well in Sanitary Department Compound, showing method of suspending tubes containing adults under a tin of water to isolate from ants.

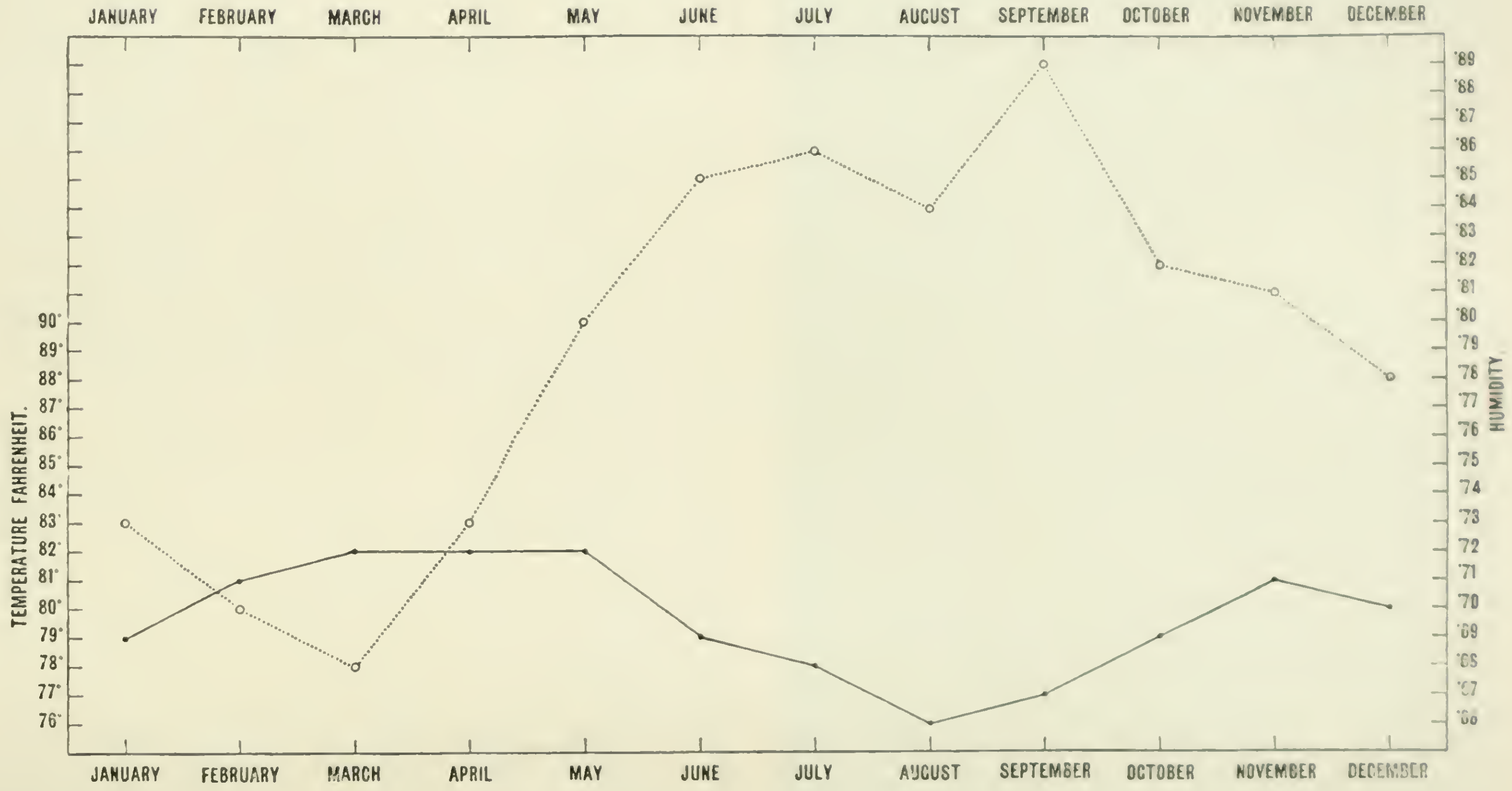
TEMPERATURE AND HUMIDITY RECORDS AT 3 FEET ABOVE WATER LEVEL IN THE WELL IN THE COMPOUND OF THE SANITARY DEPARTMENT, WATER STREET, FREETOWN.

Date.	Temperature.	Humidity.
21 December...	82° F.	...
23 ,, ...	82° ,,	·80
26 ,, ...	81° ,,	·90
18 January ...	81° ,,	·85
21 ,, ...	82° ,,	·90
25 ,, ...	80° ,,	·90
28 ,, ...	80° ,,	·90
1 February ...	80° ,,	·95
4 ,, ...	80° ,,	·95
8 ,, ...	81° ,,	·90
26 ,, ...	82° ,,	·85
3 March ...	83° F.	·85
5 ,, ..	82° ,,	·90
9 ,, ...	82° ,,	·90
13 ,, ...	83° ,,	·80
16 ,, ...	85° ,,	·76
19 ,, ...	82° ,,	·90
2 April ...	85° ,,	·72
6 ,, ...	84° ,,	·80
9 ,, ...	84° ,,	·80
13 ,, ...	85° ,,	·76
16 ,, ...	83° ,,	·90
20 ,, ...	84° ,,	·80
23 ,, ...	82° ,,	·90
27 ,, ...	83° ,,	·85

Observations made at 10 a.m.

Chart No. 8.

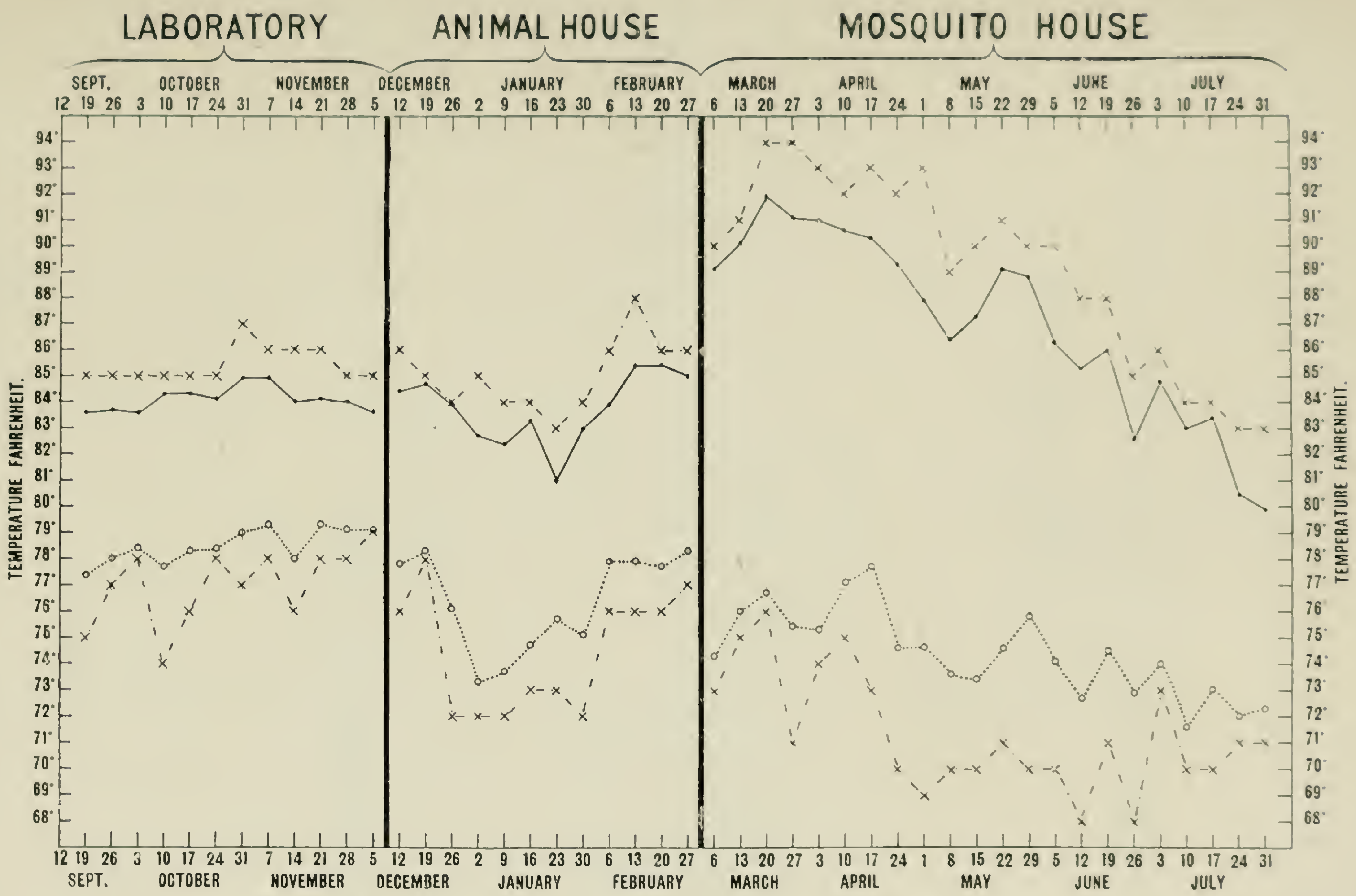
Curves of Temperature and Humidity at the Meteorological Station, Freetown, Sierra Leone (224 feet above sea level).



Observations at 9 a.m. Monthly means compiled from the Official Report for 1914.

Chart No 9.

Curves of Maximum and Minimum Temperatures and Weekly Extremes.



In the Laboratory from September 11th to December 4th; in the Animal House from December 4th to February 27th and in the Mosquito House from February 27th to July 31st.

Maximum Temperature——— Minimum Temperature . . . .
Extremes for each week— · — · — · —

The following tests were carried out in the above-mentioned situations, and the results were as follows:—

Yard at 26, Westmoreland Street.

Unfed.

141 emerged 23rd to 25th January. Period of experiment, 25th to 28th January. 71 males, 70 females.

85	lived	1 day	Average 1·4 days.
49	,,	2 days	
7	,,	3 ,,	
141			

Fed Honey.

91 emerged 16th to 18th December. Period of experiment, 18th to 28th December. 34 males, 57 females.

4	lived	5 days	All or nearly all males	Average 8·3 days.
4	,,	6 ,,		
41	,,	8 ,,		
33	,,	9 ,,		
9	,,	10 ,,		
91				

Fed Thin Syrup.

About 100 emerged 27th February to 1st March. Period of experiment, 1st to 15th March.

5	lived	1 day	Average 9·3 days.
4	,,	4 days	
3	,,	6 ,,	
5	,,	8 ,,	
24	,,	10 ,,	
6	,,	12 ,,	
9	,,	13 ,,	
56 *unfinished.*			

Ants got into the tube and carried off all that remained. To accomplish this they must have crossed a two-inch water surface or have dropped three inches from the roof of the pen on to the tube.

A Second Attempt.—114 emerged 2nd to 4th April. Period of experiment, 4th to 28th April.

5	lived	1	day	Average 8·6 days.
2	,,	2	days	
6	,,	3	,,	
2	,,	4	,,	
5	,,	6	,,	
16	,,	7	,,	
39	,,	8	,,	
15	,,	10	,,	
1	,,	11	,,	
13	,,	12	,,	
4	,,	15	,,	
4	,,	16	,,	
2	,,	24	,,	
114				

*Fed on Blood.**

15 minutes' opportunity for feeding given daily. 103 emerged 28th to 30th December. Period of experiment, 30th December to 7th February. 51 males, 52 females.

8	lived	1	day	Average 10·8 days.
6	,,	3	days	
4	,,	4	,,	
6	,,	5	,,	
14	,,	6	,,	
11	,,	7	,,	
7	,,	9	,,	
1	,,	10	,,	
6	,,	11	,,	
4	,,	14	,,	
16	,,	16	,,	
8	,,	17	,,	
2	,,	18	,,	
1	,,	19	,,	
1	,,	22	,,	
4	,,	23	,,	
2	,,	24	,,	
1	,,	35	,,	
1	,,	39	,,	
103				

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* In tests when the mosquitoes are fed on blood, allowance must be made for the average being reduced by the early death of the males.

In Cage in Room at 26, Westmoreland Street.

Fed Honey.

42 emerged 25th to 26th November. Period of experiment, 26th November to 30th December. 16 males, 26 females.

16 lived 9 days
4 ,, 10 ,,
9 ,, 13 ,,
10 ,, 15 ,, Average 12·3 days.
1 ,, 21 ,,
1 ,, 22 ,,
1 ,, 24 ,,
42

Fed Honey.

75 emerged 22nd to 24th December. Period of experiment, 24th to 30th December. 9 males, 66 females.

3 lived 3 days
32 ,, 4 ,, Average 4·6 days.
32 ,, 5 ,,
8 ,, 6 ,,
75

Hospital Compound. (Slight Shade.)

Unfed.

124 emerged 19th to 21st January. Period of experiment, 21st to 24th January. 39 males, 85 females.

9 lived 1 day
103 ,, 2 days Average 2 days.
12 ,, 3 ,,
124

Fed Honey.

173 emerged 18th to 20th December. Period of experiment 20th to 26th December. 99 males, 74 females.

8 lived 2 days
7 ,, 3 ,,
51 ,, 4 ,, Average 5·2 days.
87 ,, 5 ,,
20 ,, 6 ,,
173

Fed on Blood.

15 minutes' opportunity given each day. 106 emerged 28th to 30th December. Period of experiment, 30th December to 18th January. 56 males, 50 females.

8	lived	3	days	23 of these were males.	Average 7·2 days.
22	,,	4	,,		
8	,,	5	,,		
17	,,	6	,,		
5	,,	7	,,		
18	,,	8	,,		
20	,,	11	,,	Feeding was omitted on 11th day.	
6	,,	13	,,		
1	,,	16	,,		
1	,,	19	,,		
106					

MOSQUITO HOUSE PIT.

Unfed.

About 100 emerged 17th to 19th January. Period of experiment, 19th to 31st January. Examination for sex omitted.

2	lived	4	days	Average 6·8 days.
34	,,	5	,,	
21	,,	7	,,	
37	,,	8	,,	
3	,,	10	,,	
3	,,	12	,,	
100				

Fed Honey.

About 100 emerged 7th to 9th January. Period of experiment, 9th January to 6th February. Sexes unrecognisable.

2	lived	8	days	Average 18·1 days.
21	,,	15	,,	
27	,,	17	,,	
9	,,	18	,,	
34	,,	20	,,	
3	,,	24	,,	
2	,,	26	,,	
2	,,	28	,,	
100				

MOSQUITO HOUSE—SUSPENDED 2 INCHES CLEAR OF SOIL.

Fed Honey.

48 emerged 5th to 6th December. Period of experiment, 6th to 20th December. 22 males, 26 females.

3	lived	7	days	Average 9·2 days.
14	,,	8	,,	
10	,,	9	,,	
16	,,	10	,,	
2	,,	11	,,	
2	,,	12	,,	
1	,,	14	,,	
48				

MOSQUITO HOUSE—ON GROUND AND COVERED WITH FALLEN LEAVES.

Fed Honey.

100 emerged 18–20th December. Period of experiment, 20th December to 20th January. Within four days the ants had killed and carried away all but seven or eight. These were gradually killed off. Two managed to escape until the 9th of January.

Longest life, 20 days.

MOSQUITO HOUSE—SUSPENDED 5 FEET FROM THE GROUND.

Fed Honey.

60 emerged 11th to 12th December. Period of experiment, 12th to 22nd December. 30 males, 30 females.

3	lived	1	day	Average 2·7 days.
27	,,	2	days	
27	,,	3	,,	
1	,,	4	,,	
2	,,	10	,,	
60				

Mosquito House Pit.

Fed on Blood.*

At weekly intervals, no other food. About 100 emerged, 17th to 19th January. Period of experiment, 19th January to 8th February.

2	lived	4	days	Average 7·9 days.
23	,,	5	,,	
16	,,	7	,,	
38	,,	8	,,	
14	,,	10	,,	
2	,,	12	,,	
1	,,	14	,,	
1	,,	16	,,	
1	,,	18	,,	
2	,,	20	,,	
100				

Well in Sanitary Department Compound (about 20 to 25 feet to water level).

Lined masonry, about 6 or 8 feet, through surface soil, unlined through the solid laterite rock, diameter 5 to 6 feet. Tubes let down to within 3 feet of water.

Fed Honey.

About 100 emerged, 17th to 18th December. Period of experiment, 18th to 26th December.

There were never any dead, but tell-tale remnants of legs and wings showed that the ants were at work. All the specimens were killed and carried off within eight days.

Another attempt was made, an antiformica of suitable design being used.

About 100 emerged 13th to 14th January. Period of experiment, 14th January to 11th February.

6	lived	10	days	Average at least 17·8 days.
8	,,	13	,,	
46	,,	17	,,	
11	,,	20	,,	
20	,,	24	,,	
91				

The experiment was unfinished, as the cord rotted through, and the antiformica tube and contents were lost.

---

* Allowance must be made for the early death of the males which were present but in undetermined numbers.

Fed Thin Syrup.

About 100 emerged 24th to 26th February. Period of experiment, 26th February to 23rd March.

14	lived	7	days	Average 14·3 days.
19	,,	11	,,	
16	,,	14	,,	
43	,,	18	,,	
2	,,	21	,,	
94				

The experiment was unfinished, as the rope rotted through, and the antiformica and tubes with it were lost.

A later attempt was made, copper wire being used in place of rope.

About 100 emerged 31st March to 2nd April. Period of experiment, 2nd to 27th April.

5	lived	4	days	Average 11·3 days.
18	,,	7	,,	
47	,,	11	,,	
18	,,	14	,,	
8	,,	16	,,	
2	,,	21	,,	
2	,,	25	,,	
100				

Given One Meal of Blood, then Fed on Syrup.

About 100 emerged 24th to 26th February. Period of experiment, 26th February to 19th March.

10	lived	7	days	Average 13·4 days.
39	,,	11	,,	
3	,,	14	,,	
30	,,	18	,,	
2	,,	21	,,	
84				

Experiment was unfinished, as the rope rotted through.

Another attempt was made, wire replacing the rope.

About 100 emerged 31st March to 2nd April. Period of experiment, 2nd to 23rd April.

4	lived	4	days	Average 11·8 days.
18	,,	7	,,	
38	,,	11	,,	
25	,,	14	,,	
14	,,	18	,,	
1	,,	21	,,	
100				

ANIMAL HOUSE—SUSPENDED ABOUT 2 FEET ABOVE FLOOR

Fed Honey.

51 emerged 9th to 10th December. Period of experiment, 10th to 18th December.

4	lived	3 days	Average 5·7 days.
8	,,	4 ,,	
31	,,	6 ,,	
8	,,	8 ,,	
51			

Experiment No. XXXVI.

LENGTH OF LIFE OF MALES AND FEMALES WHEN FED ON BANANAS ONLY.

A little water was added if the banana was not juicy. Kept in a H.L.C.* cage over *wet* filter paper. Emerged 17th to 19th March.

Date of death.	Males.	Females.	Number of days after emergence.	Remarks.
20 March	1 dead	Nil	1	
21 ,,	6 ,,	1 dead	2	
22 ,,	Nil	1 ,,	3	
23 ,,	1 dead	2 ,,	4	
26 ,,	1 ,,	2 ,,	7	
28 ,,	Nil	3 ,,	9	
29 ,,	1 dead	1 ,,	10	
30 ,,	3 ,,	Nil	11	
31 ,,	6 ,,	2 dead	12	
1 April	4 ,,	...	13	
2 ,,	2 ,,	3 dead	14	
4 ,,	3 ,,	2 ,,	16	
6 ,,	1 ,,	3 ,,	18	
7 ,,	Nil	1 ,,	19	
8 ,,	1 dead	Nil	20	
9 ,,	1 ,,	2 dead	21	
10 ,,	1 ,,	2 ,,	22	
11 ,,	1 ,,	6 ,,	23	
13 ,,	Nil	2 ,,	25	
14 ,,	,,	2 ,,	26	
18 ,,	,,	1 ,,	30	
21 ,,	,,	1 ,,	33	
24 ,,	,,	1 ,,	36	
26 ,,	,,	1 ,,	38	
27 ,,	,,	1 ,,	39	
30 ,,	,,	1 ,,	42	
4 May	,,	1 ,,	46	
	33	42		

Average life: males 10·9 days; females 19·6 days.
Longest ,, ,, 23 ,, ,, 46 ,,

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* Cage made out of a hurricane lamp chimney. See illustration, page 175.

Experiment No. XXXVII.

LENGTH OF LIFE OF MALES AND FEMALES FED ON SYRUP ONLY.

Kept in a H.L.C. cage over *wet* filter paper. Emerged 17th to 19th February.

Date of death.	Males.	Females.	Number of days after emergence.	Remarks.
20 February	1 dead	Nil	1	
28 ,,	2 ,,	1 dead	9	
1 March	5 ,,	2 ,,	10	Under these moist conditions the insects are prone to get their legs smeared with syrup, and this causes a certain amount of mortality.
2 ,,	1 ,,	1 ,,	11	
3 ,,	1 ,,	Nil	12	
4 ,,	1 ,,	,,	13	
6 ,,	2 ,,	,,	15	
7 ,,	2 ,,	,,	16	
8 ,,	1 ,,	1 dead	17	
11 ,,	1 ,,	4 ,,	20	
12 ,,	3 ,,	1 ,,	21	
14 ,,	2 ,,	Nil	23	
15 ,,	4 ,,	3 dead	24	
16 ,,	Nil	15 ,, *	25	*A tiny leak in the syrup pan led to a high concentration, which may have been partly responsible for this sudden increase in the death rate.
17 ,,	1 dead	7 ,,	26	
18 ,,	Nil	1 ,,	27	
19 ,,	1 dead	3 ,,	28	
20 ,,	Nil	2 ,,	29	
21 ,,	,,	1 ,,	30	
23 ,,	,,	2 ,,	32	
31 ,,	,,	1 ,,	40	
5 April	,,	1 ,,	45	
7 ,,	,,	1 ,,	47	
9 ,,	,,	1 ,,	49	
10 ,,	1 dead	Nil	50	
21 ,,	Nil	1 dead	60	
	29	49		

Average life : males, 17·6 days ; females, 26·3 days.
Longest ,, : ,, 50 ,, ; ,, 60 ,,

Experiment No. XXXVIII.

LENGTH OF LIFE OF MALES AND FEMALES FED ON SYRUP ONLY (BOILED WATER AND TATE CUBE SUGAR).

Kept in a H.L.C. cage over *dry* filter paper. Emerged 10th to 12th February.

Date of death.	Males.	Females.	Number of days after emergence.	Remarks.
13 February	Nil	1 dead	1	
18 ,,	1 dead	Nil	6	
24 ,,	1 ,,	,,	12	
6 March	1 ,,	,,	22	
7 ,,	1 ,,	,,	23	
8 ,,	1 ,,	1 dead	24	
11 ,,	4 ,,	Nil	27	
12 ,,	Nil	1 dead	28	
13 ,,	1 dead	Nil	29	
15 ,,	Nil	1 dead	31	
16 ,,	2 dead	Nil	32	
17 ,,	1 ,,	,,	33	
18 ,,	Nil	1 dead	34	
19 ,,	3 dead	2 ,,	35	
20 ,,	1 ,,	3 ,,	36	
22 ,,	1 ,,	1 ,,	38	
23 ,,	Nil	2 ,,	39	
24 ,,	,,	2 ,,	40	
25 ,,	1 dead	Nil	41	
26 ,,	1 ,,	1 dead	42	
27 ,,	Nil	1 ,,	43	
28 ,,	,,	1 ,,	44	
31 ,,	,,	4 ,,	47	
1 April	,,	2 ,,	48	
2 ,,	,,	2 ,,	49	
4 ,,	,,	2 ,,	51	
7 ,,	,,	1 ,,	54	
9 ,,	,,	1 ,,	56	
11 ,,	,,	1 ,,	58	
12 ,,	,,	1 ,,	59	
14 ,,	,,	1 ,,	61	
16 ,,	,,	1 ,,	63	
21 ,,	,,	1 ,,	68	
	20	35		

Average life : males, 29 days ; females, 44·1 days.
Longest ,, : ,, 42 ,, ; ,, 68 ,,

Experiment No. XXXIX.

RELATIVE LENGTH OF LIFE OF MALES AND FEMALES FED ON HONEY ONLY.

Kept in a H.L.C. cage over *wet* filter paper. Emerged 24th to 25th November.

Date of death.	Males.	Females.	Number of days after emergence.	Remarks.
7 December	1 dead	Nil	12	
14 ,,	2 ,,	,,	19	
18 ,,	1 ,,	2 dead	23	
19 ,,	Nil	2 ,,	24	
20 ,,	,,	1 ,,	25	
21 ,,	,,	2 ,,	26	
22 ,,	,,	3 ,,	27	
24 ,,	2 dead	1 ,,	29	
25 ,,	Nil	1 ,,	30	
27 ,,	,,	2 ,,	32	
28 ,,	,,	1 ,,	33	
29 ,,	,,	2 ,,	34	
30 ,,	,,	1 ,,	35	
31 ,,	,,	1 ,,	36	
4 January	,,	1 ,,	40	
6 ,,	,,	1 ,,	42	
12 ,,	,,	1 ,,	48	
15 ,,	,,	1 ,,	51	
17 ,,	,,	1 ,,	53	
	6	24		

Average life: males 21·8 days; females 32·5 days.
Longest ,, : ,, 29 ,, ; ,, 53 ,,

Experiment No. XL.

RELATIVE LENGTH OF LIFE OF MALES AND FEMALES FED ON HONEY ONLY.

Kept in a H.L.C. cage, over *dry* filter paper. Emerged 3rd to 5th January.

Date of Death.	Males.	Females.	Number of days after emergence.	Remarks.
6 January	1 dead	Nil	1	
7 ,,	2 ,,	,,	2	
8 ,,	Nil	1 dead	3	
9 ,,	10 dead	9 ,,	4	
10 ,,	1 ,,	4 ,,	5	
16 ,,	1 ,,	Nil	11	
18 ,,	1 ,,	1 dead	13	
19 ,,	Nil	1 ,,	14	
20 ,,	4 dead	3 ,,	15	
21 ,,	2 ,,	1 ,,	16	
22 ,,	2 ,,	1 ,,	17	
23 ,,	5 ,,	6 ,,	18	
24 ,,	Nil	3 ,,	19	
29 ,,	,,	1 ,,	24	
	29	31		

Average life: males 10 days; females 11·4 days.
Longest ,, : ,, 18 ,, ; ,, 24 ,,

Experiment No. XII.

EGG PRODUCTION AFTER A SINGLE MEAL OF BLOOD, AND EVIDENCE OF THE EGG RETAINING HABIT, AND ALSO SHOWS COMPARATIVE LENGTH OF LIFE OF MALES AND FEMALES.

A number of males and females which emerged on the 1st to 3rd December were allowed free intercourse in a large cage. The females were given an opportunity of feeding on human blood; those that filled themselves to repletion were selected for the experiment. Twelve males and 23 females were transferred to a H.T.C. cage with *wet* filter paper at base, and supplied liberally with honey.

Date of deposition of eggs or death.	Number of eggs laid.	Fertility.	Death of males.	Death of females.	Number of days after meal of blood.	Remarks.
4 December	61	+	...	...	2	The number of eggs laid presupposes that only two or at the most three females were laying.
5 ,,	51	+	...	...	3	
6 ,,	5	+	...	...	4	
7 ,,	148	+	...	...	5	
8 ,,	4	+	...	...	6	
9 ,,	11	+	...	...	7	
10 ,,	...		1 dead	...	8	
12 ,,	5	+	...	2 dead	10	
13 ,,	4	+	...	1 ,,	11	
14 ,,	1	+	...	1 ,,	12	
15 ,,	13	+	1 dead	...	13	
17 ,,	...	...	4 ,,	...	15	
20 ,,	...	...	1 ,,	...	18	
21 ,,	41	+	1 ,,	...	19	
22 ,,	1	+	...	1 dead	20	
25 ,,	4	+	...	...	23	
26 ,,	31	+	1 escaped	2 dead	24	
27 ,,	11	+	...	3 ,,	25	
28 ,,	1	+	2 dead	...	26	
29 ,,	1	+	...	1 dead	27	
30 ,,	32	+	...	...	28	
31 ,,	4	+	...	...	29	
1 January	19	+	...	1 dead	30	
2 ,,	...	...	...	1 ,,	31	
5 ,,	170	+	...	...	34	
6 ,,	13	+	...	1 dead	35	
7 ,,	...	...	...	1 ,,	36	
8 ,,	...	...	1 dead	...	37	
9 ,,	...	...	...	2 dead	38	
11 ,,	105	+	...	1 ,,	40	
16 ,,	3	−	...	...	45	
20 ,,	...	...	...	1 dead	49	
25 ,,	...	...	...	3 ,,	54	
13 February	...	...	...	*1 ,,	73	

* The ovaries of this female on dissection proved to be fully developed and the eggs ripe for laying, many of them being already brown. It is significant that this should be the case 70 days after the only meal of blood she received.

Average life : 12 males 18·8 days ; 23 females 32·3 days.
Longest ,, : ,, ,, 37 ,, ; ,, ,, 73 ,,

Experiment No. XLII.

EVIDENCE OF EGG RETAINING HABIT AND LENGTH OF LIFE AFTER A SINGLE MEAL OF BLOOD (SUBSEQUENT FOOD HONEY).

Females which emerged on the 5th, 6th, 7th, 8th and 9th December were kept for 24 hours in a cage with a number of males, they were then allowed one full meal of blood (Nos. 1, 2, 3, 4 and 5 on the 7th December, Nos. 6, 7 and 8 on the 9th December, Nos. 9, 10 and 11 on the 10th December). Each female was then placed in a separate jar with *wet* filter paper at the bottom and fed on honey.

Date of deposition of eggs or death.	Identification number.	Number of eggs laid.	Fertility.	Death.	Number of days after meal of blood.	Remarks.
8 December	2	...	...	died	1	
10 ,,	4	69	+	...	3	
,,	5	46	+	...	3	
11 ,,	4	1	...	...	4	No record of hatching.
,,	5	1	..	...	4	,, ,, ,, ,,
,,	6	71	+	...	2	
,,	8	13	+	...	2	
12 ,,	5	1	...	...	5	No record of hatching.
,,	6	1	...	...	5	,, ,, ,, ,,
,,	8	52	+	...	3	
,,	9	1	...	...	3	No record of hatching.
,,	10	60	+	...	2	
,,	11	38	+	...	2	
13 ,,	4	4	+	...	6	
,,	6	1	...	...	4	No record of hatching.
15 ,,	11	2	...	...	5	,, ,, ,, ,,
16 ,,	3	...	...	escaped	9	Laid no eggs.
17 ,,	6	1	+	...	8	
,,	9	1	+	...	7	
,,	11	2	+	...	7	
18 ,,	7	...	...	died	9	Laid no eggs.
19 ,,	4	2	+	...	12	
20 ,,	5	...	...	died	13	
,,	9	2	+	...	10	
,,	11	2	+	...	10	
26 ,,	6	...	...	died	17	
31 ,,	10	...	...	,,	21	
1 January	11	...	...	,,	22	
2 ,,	9	1	+	...	23	
6 ,,	8	1	−	...	28	Egg infertile.
,,	9	2	+	...	28*	*These eggs resisted immersion. After three months' dry storage one of them retained its shape but did not hatch in spite of a long period in water. On the 26th April it was opened and found to contain a living larva.
10 ,,	8	...	...	died	32	
13 ,,	1	...	...	,,	37	
24 ,,	9	1	infertile	...	46	
26 ,,	4	...	...	died	50	
1 February	9	...	...	,,	53	

Average life : 25·6 days : longest life : 53 days.

Experiment No. XLIII.

TEST TO ASCERTAIN IF WELL-FED FEMALES WOULD OVIPOSIT APART FROM WET SURFACES, AND THEIR LENGTH OF LIFE WHEN EGGS HAVE TO BE RETAINED OWING TO DROUGHT.

Females that emerged between the 20th and 29th December were allowed free intercourse with a number of males in a large cage.

Those which fed readily on a human arm were selected and placed in a H.T.C. cage based on *dry* filter paper, the top covered with fine gauze; no additional cover being used to prevent evaporation.

A supply of honey was kept in the cage, and a ten minutes' opportunity of taking human blood was afforded each night.

Date of deposition of eggs or death.	Number of eggs laid.	Fertility.	Death.	Number of days after first meal of blood.	Remarks.
31 December	1	infertile	1	2	
2 January	...	...	1	4	
7 ,,	...	...	1	9	
8 ,,	...	...	3	10	
9 ,,	...	...	3	11	
22 ,,	...	...	1	24	
23 ,,	...	...	6	25	
24 ,,	...	...	1	26	
25 ,,	...	...	1	27	
29 ,,	...	...	1	31	
30 ,,	...	...	1	32	
31 ,,	...	...	1	33	
1 February	...	...	2	34	
2 ,,	...	...	1	35	
3 ,,	...	...	2	36	
4 ,,	...	...	1	37	
5 ,,	...	...	2	38	
7 ,,	...	...	3	40	
8 ,,	...	...	2	41	
11 ,,	...	...	1	44*	*This female when dying was placed in a small tube with a piece of *wet* filter paper. She immediately commenced to oviposit and laid a small batch of *infertile* eggs.
5 March	...	...	1	66	
12 ,,	...	...	1	73	
13 ,,	...	...	2	74	
			39		

Average life, 31·3 days; longest life, 74 days.

Only one infertile egg was laid on dry paper, although a dying female laid at once when provided with a wet surface.

CONCLUSION: *Water or wet surfaces* are essential to egg laying.

Experiments Nos. XLI. and XLII. It will be noted that female No. 1 lived 37 days but died without ovipositing, while Nos. 3 and 7 also failed to lay any eggs. Unfortunately, time did not permit of the dissection of Nos. 1 and 7 to ascertain if their ovaries were ripe, or if they were awaiting further meals of blood before developing them. It seems more probable, however, that the eggs were being retained.

The oviposition of females Nos. 4, 5, 6, 8, 10, and 11 was not very unusual, but that of No. 9 was distinctly abnormal, in that she dribbled out pairs or single eggs at long intervals. Those laid 23 and 28 days after the initial meal of blood proved to be fertile. Time did not permit of her dissection.

If the two experiments are considered together there seems to be a strong case made out for the instinctive retention of eggs by a proportion of the females, and this is probably the explanation why certain females in earlier experiments failed to lay, although they were afforded opportunities of sucking human blood every night.

TWO EXPERIMENTS TO ILLUSTRATE EGG PRODUCTION AND LAYING, APART FROM FEEDING ON A LIVING ANIMAL.

Attempts were made to induce egg production by feeding on mixtures of honey and white of egg, honey and dried blood, honey and fresh blood, syrup and fresh blood.

Honey and white of egg kept well in a stoppered jar for three weeks, but fermented within a day or two when placed in the cage for the females to feed on. Honey and dried blood did not keep well, possibly owing to water being used to mix the blood into a paste before adding it to the honey.

Honey and fresh blood and syrup and fresh blood kept for months when placed in a desiccator to cause a high concentration of sugar.

The first attempts with honey and white of egg, honey and dried blood, and honey and fresh blood were all negative. Some 50 to 100 females were used in each case, equal or larger numbers of males being kept with them. Pairing took place freely but the ovaries never developed, although the average life was considerable. The sole surviving female in one of the experiments, after living for 56 days, was given the opportunity of sucking human blood. She fed sparingly on three successive nights, then missed a night, and laid 21 fertile eggs on the following night. After depositing the eggs she bit but did not obtain blood, and died the next day. Two attempts, Experiments Nos. XXIV. and XXV., were then made, in one, honey and fresh blood from a sheep was again used, in the other, syrup and fresh blood from a sheep. As will be seen from the following records both met with sufficient success to be of interest theoretically, but so modified as to afford little hope of the method proving of any practical assistance in future research.

Experiment No. XLIV.

A number of males and females, which emerged on the 13th and 14th January, were placed in a hurricane lamp chimney cage on *wet* filter paper, and given an ample supply of honey mixed with blood from a sheep. This was changed on the first signs of fermentation.

Date of deposition of eggs or deaths.	Number of eggs laid.	Fertility.	Death of males.	Death of females.	Number of days after start.	Remarks.
17 January	...	...	4 dead	2 dead	3	
18 ,,	...	...	3 ,,	Nil	4	
19 ,,	...	...	Nil	1 dead	5	
20 ,,	...	...	3 dead	1 ,,	6	
22 ,,	...	...	2 ,,	Nil	8	
23 ,,	...	...	1 ,,	,,	9	
24 ,,	...	...	2 ,,	,,	10	
25 ,,	...	...	2 ,,	,,	11	
27 ,,	...	...	4 ,,	,,	13	
28 ,,	...	...	1 ,,	,,	14	
30 ,,	...	...	4 ,,	5 dead	16	
31 ,,	...	...	3 ,,	Nil	17	
1 February	...	...	7 ,,	,,	18	
2 ,,	...	...	1 ,,	,,	19	
3 ,,	...	...	6* ,,	4*dead	20	*Most of these died from mechanical causes, their legs getting fixed in the sticky mixture owing to rapid drying.
4 ,,	...	...	Nil	2 ,,	21	
5 ,,	...	...	1 dead	2 ,,	22	
6 ,,	...	...	1 ,,	3 ,,	23	
7 ,,	...	...	1 ,,	3 ,,	24	
8 ,,	...	...	1 ,,	1 ,,	25	
9 ,,	...	...	Nil	1 ,,	26	
10 ,,	...	...	,,	2 ,,	27	
11 ,,	...	...	,,	2 ,,	28	
12 ,,	...	...	,,	1 ,,	29	
13 ,,	...	...	1 dead	1 ,,	30	
16 ,,	...	...	Nil	1 ,,	33	
17 ,,	...	...	1 dead	1 ,,	34	
19 ,,	...	...	Nil	2 ,,	36	
24 ,,	...	...	,,	1 ,,	41	
25 ,,	...	...	,,	1 ,,	42	
27 ,,	...	...	,,	2 ,,	44	
28 ,,	...	...	,,	1 ,,	45	
1 March	...	...	,,	1 ,,	46	
2 ,,	1	*Infertile*	,,	Nil	47	
7 ,,	...	...	,,	1 dead	52	
12 ,,	...	...	,,	1 ,,	57	
12 April	...	...	,,	1 ,,	88	
			49	44		

Result.—1 infertile egg.

Average life : males, 14·6 days ; females, 27·8 days.

Longest ,, : ,, 34 ,, ; ,, 88 ,,

Experiment No. XLV.

A number of males and females which emerged on the 1st and 2nd March were placed in a H.L.C. cage over *wet* filter paper and given an ample supply of syrup mixed with blood from a sheep. The supply was changed on the first sign of fermentation.

Date of deposition of eggs or death.	Number of eggs laid.	Fertility.	Death of males.	Death of females.	No. of days from start.	Remarks.
4 March	1	+	...	2 dead	2	The egg hatched on 7th March.
5 ,,	...	...	1 dead	Nil	3	
6 ,,	...	...	6 ,,	,,	4	
7 ,,	...	...	1 ,,	,,	5	
8 ,,	...	...	2 ,,	,,	6	
9 ,,	...	...	3 ,,	,,	7	
11 ,,	...	..	2 ,,	2 dead	9	
12 ,,	...	...	Nil	2 ,,	10	
14 ,,	...	...	1 dead	1 ,,	12	
15 ,,	...	...	2 ,,	Nil	13	
16 ,,	...	...	1 ,,	1 dead	14	
17 ,,	...	...	1 ,,	3 ,,	15	
20 ,,	...	...	Nil	1 ,,	18	
22 ,,	..	...	2 dead	3 ,,	20	
23 ,,	...	...	1 ,,	1 ,,	21	
24 ,,	...	...	1 ,,	Nil	22	
25 ,,	...	...	Nil	2 dead	23	
26 ,,	...	...	1 dead	2 ,,	24	
27 ,,	...	...	1 ,,	4 ,,	25	
28 ,,	...	...	1 ,,	1 ,,	26	
29 ,,	...	...	1 ,,	3 ,,	27	
30 ,,	..	...	1 ,,	2 ,,	28	
1 April	...	...	2 ,,	5 ,,	30	
2 ,,	...	...	3 ,,	1 ,,	31	
3 ,,	...	...	1 ,,	1 ,,	32	
5 ,,	...	...	Nil	1 ,,	34	
6 ,,	...	...	,,	2 ,,	35	
8 ,,	...	...	,,	1 ,,	37	
19 ,,	...	...	,,	2 ,,	48	
27 ,,	...	...	,,	1 ,,	56	
30 ,,	...	...	,,	3 ,,	59	
1 May	...	...	,,	1 ,,	60	
3 ,,	...	...	,,	2 ,,	62	
4 ,,	...	...	,,	1 ,,	63	
5 ,,	...	...	,,	2 ,,	64	
13 ,,	...	...	,,	1 ,,	72	
27 ,,	...	...	,,	1 ,,	86	
			35	55		

Result : 1 fertile egg.

Average life : males, 15·6 days ; females, 32·5 days.
Longest ,, ,, 32 ,, ,, 86 ,,

Experiments Nos. XLVI. and XLVII.

FERTILITY OF MALES AND THE SURVIVAL OF SPERMATOZOA IN THE SPERMATHECÆ OF THE FEMALE (*STEGOMYIA FASCIATA*).

In these experiments, Nos. XLVI. and XLVII., the main object was to determine how many females a single male is able to impregnate and the scope of the ability to fertilize eggs which is imparted.

The longest time between pairing and the laying of fertile eggs which was observed during the progress of the research was 62 days—on the part of the female used in Experiment No. VII. This period was but a portion of her active productive life; while it lasted she laid 711 eggs in 15 batches. Subsequently she lived a further 29 days and laid 6 batches, containing 89 infertile eggs. In another experiment (No. XLI.) fertile eggs were laid 40 days after impregnation, which had been effected prior to the only meal of blood the insects received.

Neither of these cases can compare with Goeldi's classic example of the retention of spermatozoa for 102 days; nor are they, strictly speaking, comparable, because Goeldi's specimen only received the meal of blood necessary for the development of her eggs towards the close of her segregation.

Three experiments were attempted; the first was a complete failure owing to the escape of the male after pairing with two females. The second trial came to an untimely end with the death of the male after 7 days, during which period he fertilized 4 out of 5 females placed with him on successive days.

The third attempt promised better success, as the male lived 21 days and fertilized, more or less effectually, 10 of the 21 females that were placed with him. The females, however, proved to be very poor egg producers and of low vitality generally. This may have been due to underfeeding in the larval stage, as they were drawn from a very large brood at a period of the research when the amount of food required by the larvæ had not received proper attention. On the other hand, the small egg-laying capacity may have been a matter of heredity. It was certainly not due to want of food in the adult stage, as they all had nightly opportunities, and, for the most part, fed as regularly as females which laid much larger batches of eggs.

Another factor which acted to the detriment of the experiment was attacks by ants. It was only towards the close of the series that it was discovered that inequalities in the rims of some of the jars used allowed the small yellow house ants, *Monomorium pharaonis L.*, to gain entrance. The disappearance of females and possibly a considerable percentage of the mortality is to be ascribed to this cause. If, however, the second and third attempts (Experiments Nos. XLVI. and XLVII.) are considered in conjunction, a fair idea of the scope of the males' ability in the matter of impregnation will be obtained.

Some allowance must be made for mortality among the fertilized eggs, which did not perhaps receive the most favourable treatment, but this source of error was minimised as far as possible by dissecting those eggs which failed to hatch on re-immersion after three months' dry storage, the number of those which contained developed larvæ being added to those which had already hatched. In several instances *living* larvæ were extracted from the eggs, but in most cases they were dead. In doubtful cases where full development of the larvæ was not demonstrated by the presence of the egg breaker on its head, the eggs were passed as infertile.

An appended table gives the record of the eggs laid by female No. III. in Experiment No. XLVI. in full detail.

Experiment No. XLVI.

FERTILITY TEST WITH *STEGOMYIA FASCIATA.*

The *male emerged 25th October, and died 1st November.
The females after feeding were placed with the male in rotation.

Female.	Date of emergence.	Date with male.	Started to lay.	Batches of eggs laid.												Total number laid.	Number of eggs which hatched or developed to larvæ in the respective batches.												Total number hatched.	Percentage of fertile eggs.	Close of experiment.
				1	2	3	4	5	6	7	8	9	10	11	12		1	2	3	4	5	6	7	8	9	10	11	12			
I.	24 Oct.	25 Oct.	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...				5 Dec.
II.	25 ,,	26 ,,	30 Oct.	9	35	...	...	...	...	...	...	...	...	...	...	44	2	13	...	...	...		...	...	...	...	...	...	15	34	3 Nov.
III.†	27 ,,	27 ,,	30 ,,	91	86	80	59	85	86	77	84	63	53	41	32	837	86	55	73	59	63	67	76	70	51	42	4	28	680	81	3 Dec.
IV.	27 ,,	28 ,,	8 Nov.	5	28	77	...	...	...	...	...	...	...	...	...	110	4	28	23	...	...	...	...	...	...	...	...	...	55	50	14 Nov.
V.	27 ,,	29 ,,	2 ,,	15	40	84	...	...	...	...	...	...	...	...	...	139	0	0	3	...	...	...	...	...	...	...	...	...	3	2	21 ,,
VI.	30 ,,	30 ,,	14 ,,	9	...	...	...	...	...	...	...	...	...	...	...	9	0	...	...	...	...	...	...	...	...	...	...	...	0	..	15 ,,
																1,139													753	66	

* Bred from egg laid 14th September, which was immersed until 15th October without hatching, was then dried and returned to the water; it hatched the same day; the larva pupated 23rd October.

† Detailed account of hatching appended.

REMARKS.—I., died without egg laying, although she fed well at frequent intervals; II. and III., found dead; IV., killed through an accident; V. and VI., found dead.

DETAILS OF THE LAYING AND HATCHING OF THE EGGS OF FEMALE No. III.

NOTE.—The number of eggs laid each day are given, and not in batches as in the experiment itself. The method employed was to allow the eggs to incubate in glass tubes on damp paper for from 2 to 5 days, the tubes being uncorked to allow gradual drying after the second day. Eggs immersed within two days were not dried at all.

Date of laying.	Number laid.	Reference number.	Dates of hatching.																									Total number which hatched.	Percentage which hatched.	Living larvæ extracted from eggs, 29th April	Dead larvæ extracted from eggs.	Percentage of fertility.
			November.																				December.									
			1	2	3	4	5	6	8	9	11	12	13	16	17	18	19	21	26	27	29	30	2	3	4	8	15					
30 Oct.	91	1	1	30	3	40	10	2	...	...	...	...	...	...	...		...	...	...	...	...	...	...	...	...	...	...	86	95	...	...	95
1 Nov.	2	2	...	...	2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	2	100	...	...	100
2 ,,	84	3	...	...	...	1	...	46	...	...	...	...	...	...	...	...	...	...	...	...	...	...	5	...	...	...	1	53	63	...	..	63
5 ,,	80	4	...	...	...	...	...	...	54	11	...	...	...	...	...	...	...	...	...	...	...	...	1	...	1	...	...	67	84	..	6	91
8 ,,	59	5	...	...	...	...	...	...	...	...	34	24	1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	59	100	...	...	100
11 ,,	84	6	...	...	...	...	...	...	...	...	...	...	...	34	5	...	...	...	...	...	...	...	...	...	...	...	...	39	42	...	24	75
12 ,,	1	7	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0	...	...	...	...
13 ,,	2	8	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0	...	...	...	...
14 ,,	84	9	...	...	...	...	...	...	...	...	...	...	...	...	...	27	31	...	...	...	...	...	...	...	...	...	...	58	69	1	8	78
17 ,,	77	10	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	76	...	...	...	...	...	...	...	...	...	76	98	...	...	98
20 ,,	84	11	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	75	1	...	..	...	...	...	...	...	76	90	...	...	90
24 ,,	63	12	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	30	2	...	...	...	...	...	32	51	1	18	81
27 ,,	53	13	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	42	...	...	...	42	79	.	...	79
30 ,,	41	14	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	0	...	...	4	10
3 Dec.	32	15	...	...	..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	28	...	28	87	...	...	87
	837																											618	73	2	60	81

NOTES.—1. On the 4th November the batch was dried and again immersed. 3. On the 2nd December the batch was placed in the rain and afterwards immersed in cool rain water. 4. When immersed on the 8th November several hatched within 5 minutes and 54 within one hour. On the 2nd December the batch was placed in the rain and afterwards immersed in cool rain water. 5. When immersed on the 11th November 34 hatched within 2 hours. 9. When immersed on the 18th November 23 hatched within one hour.

Experiment No. XLVII.

FERTILITY TEST WITH *STEGOMYIA FASCIATA.*

The male emerged on the 1st November, and died on the 22nd of the same month. The females were fed twice or thrice before they were placed with the male, one, two, three or four being put into the cage with him at the same time and left for two or three days.

Female.	Date of emergence.	Dates with the male.	Started to lay.	Batch of eggs laid.														Total number laid.	Numbers which hatched or developed to larvæ in respective batches.														Total number fertilised.	Percentage of fertile eggs.	Close of experiment.
				1	2	3	4	5	6	7	8	9	10	11	12	13	14		1	2	3	4	5	6	7	8	9	10	11	12	13	14			
I.	30 Oct.	1–4 Nov.	7 Nov.	30	31	18	37	20	17	2	26	21	23	16	...	...	...	242	24	17	9	19	19	14	0	0	11	14	0	...	...	...	133	55	10 Dec.
II.	30 ,,	1–4 ,,	11 ,,	4	...	...	...	...	...	...	...	...	...	...	...	...	...	4	0	...	...	...	...	...	...	...	...	...	...	...	...	...	0		19 Nov.
III.	30 ,,	1–4 ,,	4 ,,	24	...	...	...	...	...	...	...	...	...	...	...	...	...	24	0	...	...	...	...	...	...	...	...	...	...	...	...	...	0		15 ,,
IV.	3 Nov.	4–6 ,,	9 ,,	71	52	63	11	69	...	...	...	...	...	...	...	...	...	266	68	48	63	9	68	...	...	...	...	...	...	...	...	...	250	94	23 ,,
V.	3 ,,	4–6 ,,	8 ,,	12	26	28	47	50	...	...	...	...	...	...	...	...	...	163	9	11	24	45	35	...	...	...	...	...	...	...	...	...	124	76	27 ,,
VI.	5 ,,	6–8 ,,	15 ,,	7	...	...	...	...	...	...	...	...	...	...	...	...	...	7	0	...	...	...	...	...	...	...	...	...	...	...	...	...	0		17 ,,
VII.	5 ,,	6–8 ,,	13 ,,	16	...	...	...	...	...	...	...	...	...	...	...	...	...	16	11	...	...	...	...	...	...	...	...	...	...	...	...	...	11	69	15 ,,
VIII.	5 ,,	6–8 ,,	11 ,,	17	36	...	...	...	...	...	...	...	...	...	...	...	...	53	1	0	...	...	...	...	...	...	...	...	...	...	...	...	1	2	13 ,,
IX.	5 ,,	8–11 ,,	15 ,,	2	54	16	7	23	24	27	29	14	6	3	6	25	...	236	1	42	1	0	0	0	0	0	0	0	0	0	0	...	44	19	12 Jan.
X.	8 ,,	11–14 ,,	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...			10 Nov.
XI.	8 ,,	11–14 ,,	14 Nov.	2	16	16	20	26	...	...	...	...	...	...	...	...	...	80	0	1	0	0	0	...	...	...	...	...	...	...	...	...	1	1	29 ,,
XII.	8 ,,	11–14 ,,	24 ,,	34	1	...	...	...	...	...	...	...	...	...	...	...	...	35	0	0	...	...	...	...	...	...	...	...	...	...	...	...	0		4 Dec.
XIII.	11 ,,	14–17 ,,	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...			30 Nov.
XIV.	11 ,,	14–17 ,,	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...			7 Dec.
XV.	11 ,,	14–17 ,,	10 Dec.	1	1	1	2	2	...	...	...	...	...	...	...	...	...	7	0	0	0	2	2	...	...	...	...	...	...	...	...	...	4	57	10 Jan.
XVI.	11 ,,	14–17 ,,	20 Nov.	24	...	...	...	...	...	...	...	...	...	...	...	...	...	24	0	...	...	...	...	...	...	...	...	...	...	...	...	...	0		21 Dec.
XVII.	12 ,,	17–20 ,,	21 ,,	1	...	...	...	...	...	...	...	...	...	...	...	...	...	1	0	...	...	...	...	...	...	...	...	...	...	...	...	...	0		22 Nov.
XVIII.	12 ,,	17–20 ,,	29 ,,	1	1	7	...	...	...	...	...	...	...	...	...	...	...	9	0	0	0	...	...	...	...	...	...	...	...	...	...	...	0		27 ,,
XIX.	15 ,,	20–22 ,,	3 Dec.	47	...	...	...	...	...	...	...	...	...	...	...	...	...	47	9	...	...	...	...	...	...	...	...	...	...	...	...	...	9	19	4 Dec.
XX.	17 ,,	20–22 ,,	25 Nov.	1	1	2	4	9	8	11	11	...	...	...	...	...	...	47	0	0	0	0	0	0	1	0	...	...	...	...	...	...	1	2	7 Jan.
XXI.	17 ,,	20–22 ,,	26 ,,	3	...	...	...	...	...	...	...	...	...	...	...	...	...	3	0	...	...	...	...	...	...	...	...	...	...	...	...	...	0		20 Nov.
												Total	...					1,204										Total	...				584 = 40 per cent.		

REMARKS.—I. to III., found dead; IV., disappeared, ? carried off by ants; V., escaped while feeding; VI. to XV., found dead; XVI., found dead, eggs abnormally small; XVII., disappeared, ? carried off by ants; XVIII. and XIX., found dead; XX., disappeared, ? carried off by ants; XXI., ants caught in act of devouring her.

Experiment No. XLVIII.

ENEMIES IN THE ADULT STAGE.

The most serious dangers to which adults of *Stegomyia fasciata* are exposed are probably those which arise from climatic causes. The daily rise of temperature, even in fine weather, is by no means excessive in Freetown, but it is sufficient to impart a considerable drying capacity to the air. During the dry season the dangers of desiccation are increased, while the incidence of the harmattan periods with strong, dry N.E. winds causes an enormous mortality among captive mosquitoes. Wild specimens, however, by seeking refuge either in crannies and crevices, or among moist vegetation out of doors or in dark recesses and corners within buildings, near floor level, away from any draught, can make local adjustment in their resting situations to minimize the danger. It is relative to this habit of seeking cover that the attacks of predaceous foes are most likely to occur, and, as a consequence, wingless enemies, such as toads, lizards, spiders, ants, scorpions and, possibly, also young mantids, probably take a heavier toll than do birds or flying insects, such as dragon flies; although the last named will almost certainly account for a certain number at the period when the mosquitoes leave the water and first take wing.

The habit which is so noticeable in resting *Stegomyia* of raising the third pair of legs and keeping them in constant motion, is probably a safeguard against attacks by wingless enemies, more especially ants. The elevation and movement of these legs will prevent their being easily seized hold of and they are available for an immediate kick-off, kangaroo fashion, at the slightest touch. When *S. fasciata* are deprived of their wings it at once becomes evident how considerable their leaping powers are. Two species of ants, *Monomorium pharaonis* L. and *Solenopsis geminata* F., proved themselves to be most inveterate enemies to captive mosquitoes during the progress of the research, and, as they appeared omnipresent in Freetown, mosquitoes are probably only safe from their attacks when actually flying.

In the mosquito house a small species of web-spinning spider, *Uloborus feniculatus*, and a larger species, belonging to the genus *Araneus*, which did not spin a regular web, took a heavy toll of the *S. fasciata* which were breeding in it. These spiders found entrance, when very small, through the 16 × 16 wire gauze and colonized it, breeding inside, using the corners and angles and covering the surface of the sides with their webs. The mosquitoes certainly formed their staple food, and, so far as observation of the captives in their webs went, were their sole source of supply.

Another uninvited guest in the mosquito house was a small species of scorpion, *Isometrus maculatus*, much flattened and with very slender claws. This must have found entrance through the crevice of a close-fitting, bevelled door when young, and possibly bred inside the house. From the slender, delicate structure of its pincers it is probable that it feeds on small insects only. A specimen kept in a 2-inch entomological glass-bottomed box was fed entirely on active adults of *S. fasciata*. It would capture and eat the entire number put into the box each night; sometimes disposing of 50 or 100 in a single night and, apart from its moulting periods, seems to have an insatiable appetite.

Small mantids in their first or second instars found their way into the mosquito house and were also found to capture and greedily devour specimens of *S. fasciata* when confined with them in a small box.

Two species of wall hunting lizards were also tried; both were very young, considerably less than half grown. One, a species of *Lacertidæ*, probably *echinata*, cleared a large cage of *S. fasciata* with great despatch, but the other, a baby specimen of a gecko, probably *Hemidactylus brookii*, or one of its relations, only ate occasional specimens when they were placed in a small box with it, and made no appreciable difference to the numbers of mosquitoes in a large cage.

IV. PARASITES.

The larvæ and pupæ of *Stegomyia fasciata* reared in the mosquito house were found to be heavily infected with a species of gregarine, which has subsequently been identified by Dr. C. M. Wenyon as *Lancastriara culicus* Wenyon. This gregarine was found by Mr. A. C. Stevenson and Dr. C. M. Wenyon in specimens of *S. fasciata* reared from eggs laid upon leaves which were forwarded from Freetown for exhibition at one of the meetings of the Yellow Fever Commission.

The life cycle of this species has been thoroughly worked out by Wenyon (1911) in specimens of *S. fasciata* captured and bred at Bagdad, a fully illustrated account being published in "Parasitology," Vol. IV. 1911. From Stevenson and Wenyon's account of the gregarines in the specimens reared from the Freetown eggs and the experiments carried out by myself at Freetown, it is evident that the sporocysts are able to resist desiccation, and, like the eggs of the mosquitoes they infect, lie over the dry season in the dried-up water holes, awaiting ingestion by the larvæ which hatch out at the commencement of the rains. This habit of the parasite supports the view that *Stegomyia fasciata* relies chiefly, if not solely, upon the egg stage for the survival of the species during the dry period, as there would be no need for the sporocysts to have developed the ability to resist desiccation if the adult host survived the dry season in any numbers.

A trial carried out in order to test if infestation by the parasites had any checking influence on the development of the mosquito, or caused any mortality during larval life, showed no evidence of any ill effect on the development of the insect, nor was there any noticeable mortality in the larval stage. If any deaths at all occurred among the infected larvæ it was confined to the first or second instars.

The only other organisms detected in *S. fasciata* during the research were bacteria, which were present in small numbers in the larval gut and more plentiful in the stomachs of the adults, and some small oval bodies of from 2μ to 4μ in diameter, which were present in the stomachs of the adults. These were first noticed in smears of the dissected gut stained with Giemsa, and were suspected of belonging to the *microsporidia*, but sections failed to reveal any intracellular phases of the life cycle, nor could any undoubted evidence be obtained of their occurrence other than in the lumen of the gut. My colleague, Dr. H. M. Woodcock, of the Lister Institute, pronounced them to be yeasts.

V. STERILITY OF THE PUPAL GUT OF THE *STEGOMYIA FASCIATA*

In addition to the microscopic examination of the larval and pupal gut described on page 127, bacteriological tests were commenced in collaboration with Dr. G. G. Butler. Unfortunately, the trials had to be discontinued at an early stage of the investigation, owing to the calls of other duties on Dr. Butler's time.

A living pupa was allowed to swim for a few minutes in each of six tubes of sterilized distilled water—its transference from tube to tube being made with a sterilized platinum loop. The following tests were made in tubes of broth :—

Result.

(*a*) An inoculation of water from the sixth tube ... *Sterile.*

(*b*) The living pupa was allowed to swim in a tube of broth for five minutes ... ... *Sterile.*

(*c*) The pupal gut with the malpighian tubes attached, was dissected out of the pupa and placed in a tube of broth ... ... *Sterile.*

(*d*) The pupal envelope was placed in a tube of broth *Sterile.*

Living pupæ were placed in a 1 per cent. lysol solution and allowed to swim about ; they were all killed or rendered incapable of movement within fifteen minutes, but some became quiescent much sooner than others :—

Result.

(*a*) A pupa was placed in 1 per cent. lysol for two minutes, its stomach was then dissected out and placed in a tube of broth... ... ... *Sterile.*

(*b*) The malpighian tubes of the same pupa were placed in another tube of broth ... ... *Sterile.*

(*c*) A pupa was placed in 1 per cent. lysol for five minutes, when it appeared to be dead ; it was then transferred to a tube of broth for fifteen minutes and then removed ... ... *Sterile.*

(*d*) A pupa that had been in 1 per cent. lysol for two minutes was dissected and the gut, with its appendages, placed in broth ... ... *Sterile.*

Living pupæ were placed in 1 per cent. lysol for two minutes and then washed in sterile water :—

	Result.
(*a*) The gut was dissected out of the imago, which was ready to emerge from one of these pupæ, and placed in a tube of broth	*Growth of unidentified bacilli.*
(*b*) The gut was dissected out of a pupa, in which the imaginal development was not so advanced, and placed in a tube of broth	*Sterile.*
(*c*) An entire undissected pupa was placed in a tube of broth	*Growth of unidentified bacilli.*
The gut was dissected, with the usual precaution of sterilized needles, slides and salt solution, from an unsterilized pupa, and placed in a tube of broth	*Sterile.*

It seems more probable that the pupal gut is sterile, and that the cases of infection were due to some mischance in the technique than that the infecting organism had persisted in the gut from the larval period. Such a conclusion is supported by the microscopical examination of other pupal guts already alluded to. (*See* Experiment No. XXVII.).

VI. NOTES RELATING TO THE LIFE HISTORY OF SOME NEAR ALLIES OF *STEGOMYIA FASCIATA* AND ASSOCIATED SPECIES.

Stegomyia sugens.

Larvæ of this species were obtained solely from rock pools.

The development of the larvæ is very rapid under favourable conditions, rigorous selection, consequent upon the habit of breeding in the smallest of rock pools, probably having compressed the larval period into the shortest space consistent with physiological requirements. In captivity the eggs incubate within two days.

Eggs.—(Fig. 1, page 18). The eggs, although very similar to those of *S. fasciata* in general appearance, are smaller, shorter, and more oval in outline, with traces only of the flattening side which gives the egg of *S. fasciata* its asymmetrical aspect. The average size of six eggs was ·573 mm. in length and ·176 mm. in width, the smallest being ·530 mm. by ·176 mm. in width, the largest ·590 mm. by ·180 mm. The surface is covered with the same delicate cellular reticulation, central bosses not noticeably different from those on the eggs of *S. fasciata* being present. The wall surrounding the micropylar area was a trifle more prominent in the eggs compared, and had a slight outward slope or overhang.

As regards the power of hatching within a few minutes of immersion, serial hatching, resistance to drought and response to cooling the eggs are, so far as the smaller amount of experimental work entitles one to say, as well and similarly endowed as those of *S. fasciata.* (See experiment which follows).

Hatching of Eggs of Stegomyia sugens.—Two females reared from larvæ taken in a rock pool at Murray Town. Emerged on or about 18th October, placed in a large cage with males and afterwards kept in a small jar. One female died after the first batch was laid, the second on the 8th November.

Date on which eggs were laid.	Number of eggs laid.	Date of hatching.													
		October.		November.									December.		
		29	30	3	11	13	19	20	21	22	23	24	12	20	21
27 October	100	5	3	5	3	...	7	2	1	7	8	1	1	34	4
4 November	20	...	...	..	...	2	...	...	...	...	...	...	...	2	3

Notes.—A portion of the first batch of 100 were immersed at once, while about one-third were allowed to dry and immersed a week later. When these dried eggs were immersed 5 hatched within 15 minutes Subsequently both portions were treated alike.

The eggs were kept immersed from the 3rd to 11th November without any hatching; they were then transferred to fresh water 2° F. below that they were in. Three hatched during the night.

On the 17th November a portion of the eggs were dried, placed in an incubator at 98° F. for several hours and thence into a desiccator; on the following day they were placed in a moist atmosphere and after 24 hours drops of water were gradually added, until the filter paper on which they were laid was saturated. They were then submerged in shallow water. 7 emerged within 30 minutes.

A second portion was dried without the use of incubator or desiccator, but otherwise treated similarly. None of the eggs hatched within several hours of their immersion.

A third portion was kept immersed without any alteration of the conditions. None hatched. On the following day the pan containing these eggs was placed in an ice chest, and the temperature of the water lowered by 6° F. One larva emerged within an hour and a second within three hours.

On the following day the cooling was repeated without effect but two emerged the next day. Also 5 hatched of the batch that had been gradually wetted and immersed, after drying without the use of incubator or desiccator, three days previously.

On the 20th December, all the eggs having been kept in water since the 12th December, they were lifted from the water and exposed to the very dry wind then blowing. After half an hour they were re-immersed in the same water. 26 hatched within 15 minutes and 8 more within the hour.

These larvæ fed up rapidly and produced normal adults, which fed, paired and laid eggs in due course.

The remaining unhatched eggs were dried on the 31st December and stored on the ground in the mosquito house until April when they were re-immersed. None hatched, and on the 12th May the few remaining eggs that retained their shape were dissected and found to contain dead larvæ. These eggs, like the stored eggs of *S. fasciata*, suffered from the attacks of *Psocidæ*.

It is possible that the humid storage does not suit *S. sugens* eggs, which, judging by the situation of the breeding pools, must be capable of withstanding great extremes of heat and drought. Dried eggs have hatched within 15 minutes of their immersion, and the larvæ have grown to full size within three days. Pupation has occurred within four-and-a-half to five days, and adults have emerged within six days of the hatching of the egg.

Probably under favourable conditions in the natural state the time would be shortened, in fact, the appended note shows that it may be, but the specimens in this case were undersized.

Breeding of Stegomyia sugens in Small Rock Pools.

On the 5th May a small basin-shaped rock pool on Tower Hill (Plate No. 5, page 9) was found to be swarming with larvæ of *Stegomyia sugens*. The pool, which was capable of holding about one litre, had a small amount of muddy sand at the bottom, and was fully exposed to the sun, situated on a large bare convex rock.

The larvæ were all of a size either already in their third skin or about to moult for the second time. As from its size and shape the pool did not seem capable of resisting more than two successive dry days, it was kept under observation. The following day was sunny, and by 5 p.m. more than half the water had evaporated. As a number of *S. sugens* were required in order to obtain eggs, about half the larvæ and water was taken. (Within a week 88 male and 72 female specimens of *S. sugens* had emerged, also 3 females of *S. fasciata* and 1 of *S. luteocephala* from the larvæ taken from this pool.) It threatened to rain the same night, but the tornado passed with only a few drops of rain. The next day being fine and sunny, the pool was dried and the sand at the bottom got a thorough baking, the shade temperature being 85° F. On the next night, the 8th, it rained steadily until daybreak and the pool was visited about 5.30 p.m., when it was found to contain numbers of young larvæ full grown in their first skins. There seems no doubt that these had hatched since the rain, as, apart from the baking which the pool had on the 7th, the fact that the larvæ were younger than those found in the pool earlier precludes any possibility of their being survivors of that brood. It is further certain that they must have hatched from resistant eggs lying in the hollow, as the interval between the commencement of the shower and the finding of the larvæ was not long enough to allow of the incubation of freshly laid eggs. On the evening of the 11th the water was again very low; development had, however, been extraordinarily rapid, a few of the larvæ having already pupated (within three days of hatching), but they were distinctly small for the species. On the 12th the water had evaporated, but both larvæ and pupæ were found to be alive in the wet mud and became fully active in water. Heavy rain fell on the night of the 12th and saved the survivors from the fate of the earlier hatched members of the brood.

Adults.—No wild adults were obtained or seen. Experiments with bred specimens reveal it as an equally greedy and persistent feeder on human blood as its cogener *S. fasciata.* It, has, however, many traits which suggest it to be less domesticated than *S. fasciata*, which shows the laxness of pairing and feeding habits which so frequently occurs with animals brought into close relationship with man. Mating could only be obtained by the use of a large cage ; it was not witnessed, and apparently only took place at night, while feeding during the daytime could only be induced by darkening the cage. Feeding was, however, quite dissociated from pairing, as virgin females kept in small boxes fed readily and repeatedly, sometimes to such excess that they voided the surplus blood, apparently quite undigested. Under these conditions small numbers of infertile eggs were laid, and the length of life of some of the specimens was over a month.

A subsequent trial with a larger number, including males, was made at the close of the rains, the insects being kept in a large cage because fertile eggs were not obtained when they were kept in small cages. In this case the length of life was shorter, there being a very heavy mortality within a few days of emergence. Not more than two batches of eggs were laid by segregated females, but this does not probably represent the limits of fecundity. Feeding would seem to be adjusted in relationship to egg laying, as with *S. fasciata*, taking place on successive nights, but being refused just prior to oviposition. No exact figures as to the number of eggs laid by a single female were obtained, but two females laid over 100 in a single night.

Stegomyia simpsoni.

Larvæ of this species were obtained from water holes in a cottonwood tree, and a number were reared from a mass of leaves taken from the same holes during the dry season. A very general breeding place would, however, seem to be in small collections of water in the axils and whorls of leaves of the coco yam, cock hat tree, a species of *Dracæna* (*see* Plates 16 and 17, page 17), and banana.

Only infertile eggs were laid by captive females. (*See* Fig. 1, page 18.) These, while generally similar to those of *S. fasciata* and *S. sugens* in colour, shape, size and surface structure, differ in several minor points. They are smaller and slightly more slender than those of *S. fasciata* and bilaterally symmetrical, while the micropylar wall is lower and less evident than that of *S. fasciata*. The measurement of two or three eggs gave the size as about ·550 mm. in length by ·120 mm. in thickness.

In the absence of fertile eggs laid by captive females it was not possible to follow up the larval life in detail, or carry out any experiments regarding the endowments of the eggs in respect of resting, serial hatching and response to external *stimuli*. That the eggs are drought-resisting, and that the species depends upon tiding over the dry season in the egg stage seemed obvious, but the record of rearing from leaves stored dry (see Notes on page 148) sets the fact beyond doubt. Time did not permit of searching the leaf axils of plants during the dry season for resting eggs. Failing their discovery in such situations, it remains to be proved whether the species uses these situations as auxiliary to water holes in trees, or if it can exist indefinitely when dependent upon leaf collections of water alone.

Adults.—The insects fed readily at night on human blood and to a limited extent in a darkened cage by day. They were individually almost, if not quite, as greedy as *S. sugens* or *S. fasciata*. As only infertile eggs were laid and these only at long intervals, though in fairly large batches, it is to be presumed that pairing did not take place in spite of the fact that various expedients were tried, and that a number of both sexes were kept together in a large cage. No attempts at pairing were witnessed. The length of life was not determined with any accuracy, but some of the females certainly lived for more than a month during September—October.

Stegomyia luteocephala.

This species was obtained in the larval stage chiefly from water holes in the roots of cottonwood trees, though a few were taken in rock pools of similar size to those affected by *S. sugens*.

Collections of water in tins or other utensils yielded larvæ at rare intervals, while a single specimen was reared from the coco yam.

Eggs.—(*See* Fig. 1, page 18). Infertile eggs approximate closely to those of *S. fasciata*, but are slightly smaller than the average eggs of this species. The girth is greater in relation to the length and the taper to the ends is consequently more marked, while the flattening of one side, which gives a slight asymmetrical outline to

S. fasciata from certain aspects, is accentuated. In the particular eggs compared there was a slight difference in the cellular reticulation, the walls in *S. luteocephala* appearing higher in relation to the central bosses, while the micropylar wall was lower. In the absence of fertile eggs no experimental work was carried out, but on the evidence obtained by rearing the species from a collection of dried leaves taken from a root hole it is possible to say that the eggs are undoubtedly endowed with similar drought-resisting powers to the other species of the genus already dealt with. On two or three occasions considerable batches of infertile eggs were laid by bred females on wet filter paper or wet wood (this latter seemed more attractive), but the majority of the females either died without ovipositing or laid only a few eggs at irregular intervals.

The larvæ, like those of *S. fasciata*, appear able to survive without water if kept moist. A larva was found among wet leaves in a small water hole ; its skin was already dry where exposed to the air, and it was incapable of movement. On immersion it recovered and a male specimen was reared.

Adults.—Like the other species of *Stegomyia* the females fed readily and vigorously on human blood, and were less disinclined to bite by day in a darkened cage than either *S. simpsoni* or *S. sugens*. Numerous attempts were made to induce the insects to pair, glass jars as well as small and large wooden cages being used. No attempts at pairing were observed, and it is to be presumed, as infertile eggs were laid on several occasions by the females, that copulation did not take place. The length of life was not accurately determined, but the females lived in captivity for several weeks.

Ochlerotatus apicoannulatus.

Specimens were reared from larvæ taken in water holes at the roots of trees, in rock pools and occasionally from water in tins. Many attempts were made to pair the insects and obtain eggs. Although the insects lived for two or three weeks in captivity, and the females bit fairly frequently, it was only occasionally that they filled themselves with human blood. In several instances, however, the same individuals fed several times. Towards the close of the dry season specimens were reared from a collection of fallen leaves taken from water holes at the roots of a cottonwood tree. These were placed in a large cage kept dark and moist by hanging wet clothing over it, and one or two of the females fed greedily by day on a human arm.

No attempts at pairing were observed and no eggs were laid.

Ochlerotatus minutus.

This species was reared from larvæ taken in numbers from rock pools, old tins and water holes at the roots of trees towards the close of the rains. Numerous attempts were made to get the insects to pair and oviposit, but they were unsuccessful. The females bit frequently, and sometimes fed well on human blood.

Uranotænia ornatus.

This species was reared from larvæ taken exclusively in the water which collects in the whorls and axils of the leaves of plants ; many attempts were made to feed, pair and obtain eggs, but they were all unsuccessful, and the insects never lived more than 36 hours in captivity. The females never attempted to take human blood or betrayed the slightest inclination to bite, nor were they observed to feed on the honey provided for them.

Eretmopodites.

Three species of this genus were taken in the larval stage ; *E. quinquevittatus* from collections of water in tins, etc., in some numbers ; *E. chrysogaster* sparingly from the same situations ; and *E. draconis* commonly from collections of water in the whorls and axils of the leaves of various plants. The close similarity in general appearance and colouration of the species is very puzzling, and makes the segregation of active individuals for pairing purposes a matter of chance. The males of *E. chrysogaster* are easily determined by the tufts on their hind legs, but as regards *E. quinquevittatus* and *E. dracænæ* the only easy mode of determination is by an examination of the pupa. The females of *E. quinquevittatus* and *E. dracænæ*, and possibly those of *E. chrysogaster*, fed at irregular intervals on human blood until their stomachs were distended to an extent which greatly detracted from their elegant beauty. A number of specimens bred from a collection of larvæ were kept in H.L.C. cages and given nightly opportunities of feeding on human blood, honey being placed in the cages for the males. Occasionally they were all turned loose for a few nights into a large cage, the females being segregated afterwards for oviposition. Most of the specimens were *E. quinquevittatus*, but males at least of *E. chrysogaster* were also present. A small number of eggs were laid in twos and threes ; although there was no difference in colour or structure, there was a striking divergence in shape and size ; and as they segregated into three sizes the eggs were reared in separate jars, but the pupal stage showed conclusively that all the eggs were all those of *E. quinquevittatus*.

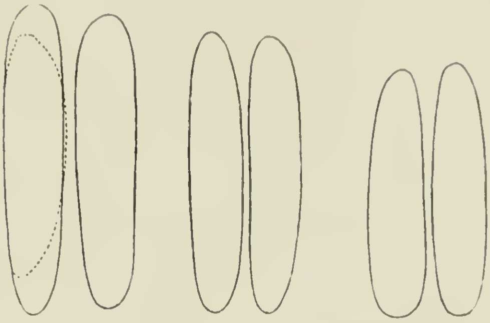

FIG. 3.

Outlines of eggs of *Eretmopodites quinquevittatus*, showing the wide variation in size which exists in the eggs of this species. Adults were reared from all the eggs. The dotted outline shows an egg of *Stegomyia fasciata* for comparison.

Both sexes were reared from the short eggs, and a pairing was watched in a H.L.C. cage ; the male came to the female while she was resting. The blood-sucking propensities of the bred specimens were as erratic as those of their parents, and biting was more frequent than actual feeding. Though the insects were hardy and bore confinement well, they did not live long—about three weeks being the average time, the longest life not being more than a month.

Eggs of E. quinquevittatus.—These were almost invariably laid horizontally, on the sides of the jar or on pieces of wood, just beneath the water surface, and the firmness of their attachment suggested the use of some cement. A small batch laid on the 12th October was divided, some of the eggs being dried and the others immersed in a pan of water. Those taken from the water all collapsed on drying while the others hatched. Unfortunately, no opportunity of repeating the experiment occurred, but the suggestion is that the eggs of this species are not drought resisting, and this is possibly supported by the fact that all the eggs previously laid hatched within a few days of laying, there being no cases of serial hatching or resistant eggs.

The eggs differ markedly in appearance from those of *Stegomyia* ; they are bright brown in colour, the shell looking thinner and more delicate. In shape they are longer and more cylindrical, with rounded rather than pointed ends, only the slightest trace of asymmetry being discernible at the micropylar end. The large eggs are nearly a third longer than those of normal *S. fasciata*, about ·8 to ·9 mm.; the medium sized about one quarter larger, about ·750 to ·780 mm., while the small eggs are scarcely, if at all, larger than normal eggs of *S. fasciata*—being about ·630 to ·650 mm. in length. These short eggs were, however, thicker than the medium sized ones, and relatively thicker than the long ones. Actually they were not quite so thick. The surface is smoother than that of the *Stegomyia* eggs, the cellular reticulation being more delicate, while the central area of the cells is slightly raised, or puffed, to use Mitchell's term. The appearance of the empty shell suggests, however, that the raised areas are structural features of the egg-shell, and not pockets containing a separate substance as in *Stegomyia.* In the absence of sections, however, it is not possible to speak with certainty on this point. All three forms of egg produced normal adults, the only observed differences being in regard to date of hatching and development of larvæ, the short eggs taking five or six days to hatch as against two or three in the case of the larger eggs; while the larvæ which emerged from them fed up more rapidly, but as the larvæ were kept in separate jars this may have been due to variation in food.

Larvæ.—The larvæ of these species are long, slender, even in comparison with those of *Stegomyia*, and of pale colouration. They show traits of ground feeding, moving over the sides and bottom of the jar with a walking or gliding movement, browsing as they go. This habit is also very noticeable with young larvæ of *Stegomyia fasciata*, but it gradually becomes less noticeable as they grow older, whereas it is characteristic of the larvæ of *Eretmopodites* when fully grown.

Pupæ.—In this stage *Eretmopodites* exhibit some marked divergencies from other mosquito pupæ, and also vary widely within the group. The abdomen is larger and longer in relation to the thorax and less curved, rendering the pupæ as a whole straighter and less "comma"-like than the other mosquito pupæ dealt with in this Report. The paddles are relatively small and heavily fringed with hairs, while in *E. quinquevittatus* the reduction in size is so marked, and the importance of the hairs on the apex of the paddles and terminal segments of the pupæ are so exaggerated, that it appears, superficially at any rate, to differ more from its fellow species within the group than the genus as a whole does from *Stegomyia* in respect of pupal characters. (*See* Figures on pp. 142 and 144.)

Adults.—As already noted, these extremely beautiful and elegant insects are certainly blood suckers. It is, however, doubtful if man is the normal host. Specimens given honey and water and banama lived for two or three weeks, although they were never observed to feed on the foods given them. They have a characteristic habit of wandering about and carefully probing with their slightly curved proboscis the surface of wood and glass, as though searching for minute drops of moisture.

VII.—NOTES REGARDING THE POSSIBILITIES OF THE PUPAL PADDLES (ANAL PLATES OF WESCHÉ) FOR PURPOSES OF CLASSIFICATION AND THE SEPARATION OF ILL-DEFINED SPECIES.

An examination of the pupa cases of the species of *Stegomyia*, *Eretmopodites*, and other mosquitoes bred at Freetown during the course of the research, showed that very marked differences may occur in the pupal paddles of closely-related species. Though time did not permit of a full examination and detailed description being made of all the pupal characters, the divergence in shape and structure of these appendages* proved so convenient and easy a method of separating species closely resembling each other as do the females of *E. quinquevittatus* and *E. chrysogaster* that preparations and camera drawings were made of as many species as possible.

Incidentally this led to the discovery of a new species bred from collections of water in the central whorl and axils of the leaves of plants, which is described by Mr. F. W. Edwards under the name of *Eretmopodites dracænæ* Edw. in the "Bulletin of Entomological Research," Vol. VI., Part 4.

Although the divergence in structure between the paddles of *E. quinquevittatus* and those of the other species of the genus *Eretmopodites* is so remarkable, this would seem to be unusual, a slight difference, such as that existing between the various species of *Stegomyia* being more general; while, on the other hand, there may be a close approximation in character, as is the case with *Anopheles (Pyretophorus) costalis* and (*Myzomyia*) *funestus*. There seems, therefore, no likelihood, so far as the scanty material examined goes, that these characters could be made to afford the basis for an independent scheme of classification, such as has already been suggested on larval characters. On the other hand, these pupal appendages, which are obviously likely to be adaptive rather than ancestral in character, afforded so ready a means of discrimination in the above case that it seems worth while to call the attention of workers in this group of insects to their possibilities.

This appears to be the more necessary as Wesché (1910) in his paper dealing with the larval and pupal stages of the West African

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* Wesché (1910) terms these fins "Anal Plates." The authors of the Carnegie Monograph (1912, etc.) refer to them as "Anal Paddles."

Culicidæ has scarcely done justice to the pupal characters. The species he treats of do not show any such striking differences as those between the various *Eretmopodites* referred to above, but, judging by the plates illustrating his article, insufficient magnification has been used and the representation of *Anopheles (Pyretophorus) costalis* is seriously in error as regards the character of the terminal hair or seta on the paddle, which is really strongly reminiscent of a boot-hook. (Compare Fig. 19, page 146, with fig. 6, plate VI., in Wesché's paper.)

It is somewhat remarkable also that Messrs. Howard, Dyar and Knab (1912) in their monograph of the Mosquitoes of North and Central America and the West Indies have made but slight use of the pupal paddles, while to my great disappointment, the magnificent plates illustrating this work show the pupæ only in lateral aspect.

Pending the description and figuring of a wider range of species, the characters afforded by the paddles can only be used in the same limited manner as at first supplied to the genitalia of insects generally, *i.e.*, as a test of separation in difficult cases. For this purpose the paddles of mosquito pupæ are readily available, as the pupa case can be examined under a low magnification and the paddles are all in one plane.

Permanent mounts are very easily made. The empty pupa case should be removed from the breeding jar as soon after the emergence of the adult as possible ; while it still floats is best. It is then spread on a slide, the wing, leg and thoracic portions of the case being separated and opened out if desired. A cover slip is dropped over it and tied down with fine cotton ; it can then be dehydrated in alcohol, transferred to xylol to clear, the thread cut off and balsam run in without disturbing its arrangement.

All the figures are drawn to the scale of 30 diameters, except that of *Toxorhynchites brevipalpis*, which, owing to its large size, has only been magnified x 10.

In several cases the setæ, though represented as simple, are actually branched, but so minutely that it was impossible to show this feature without gross exaggeration. The marginal fringes to the paddles would appear in all cases to be the development of a serrated edge, even when the fringe is very long, as with *Eretmopodites chrysogaster ;* the distinction between these tapering filaments and the round sectioned setæ on the abdominal segments, or the terminal ones on the paddles, is quite clear. In some cases, as with *Stegomyia sugens* and

Ochlerotatus apicoannulatus, the serrations are excessively minute and delicate. In most instances it was only practical to represent these fringes by single lines.

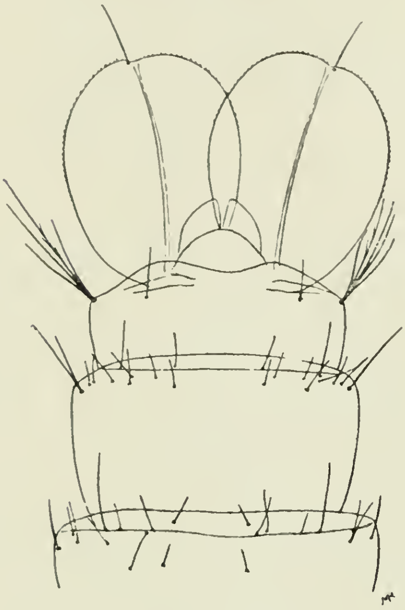

Fig. 4.

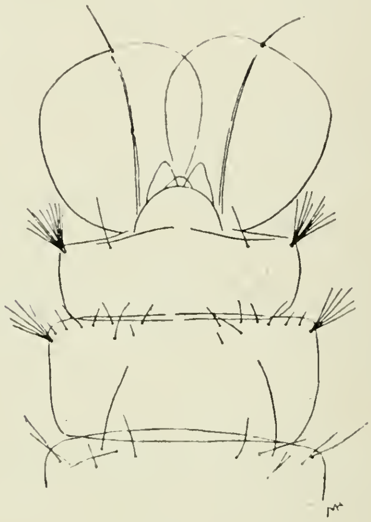

Fig. 5.

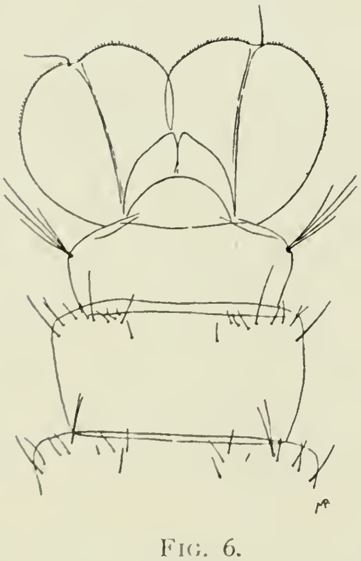

Fig. 6.

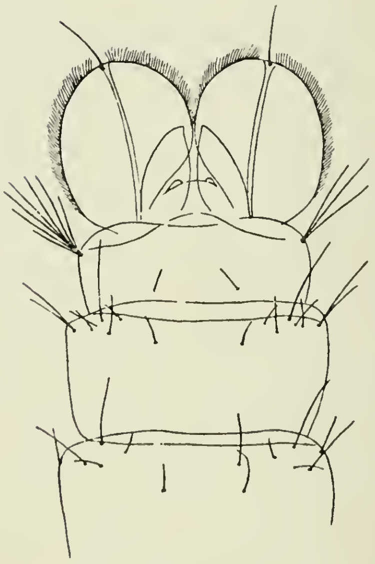

Fig. 7.

Figures 4, 5, 6 and 7 represent the paddles of *Stegomyia fasciata, sugens, simpsoni* and *luteocephala*. All four species show well marked differences, the marginal fringe in *fasciata* and *simpsoni* consists of delicate serrations. The asymmetry of the terminal lateral setæ on the eighth abdominal segment of *luteocephala* is rather noticeable, but is of course not a normal feature — asymmetrical divergence in the setæ of insects being by no means an uncommon phenomenon.

The paddles of *Ochlerotatus simulans* (Fig. 8), *apicoannulatus* (Fig. 9), and *minutus* (Fig. 10), though very similar in general appearance, each show one or more distinguishing features.

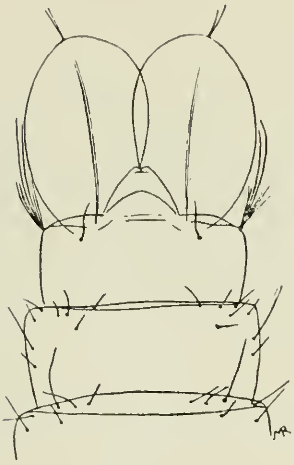

Fig. 8.

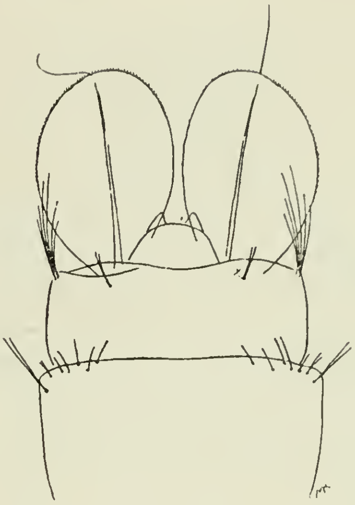

Fig. 9.

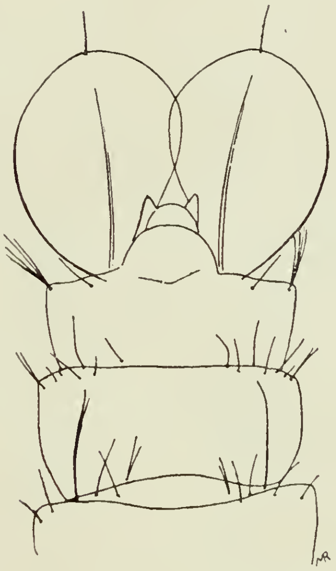

Fig. 10.

The four figures of the paddles of *Eretmopodites* are those of *quinquevittatus* (Fig. 11), *chrysogaster* (Fig. 12), *dracænæ* (Fig. 13), and an undetermined specimen (Fig. 14). The heavily fringed paddles of the last three specimens, together with the extraordinary specialization of *quinquevittatus*, make the group a very interesting one as regards this character. Only a single specimen of the undetermined species was obtained, and, owing to some mischance, the adult which emerged from it either escaped or was mislaid, and a specimen of *E. dracænæ* was by mistake associated with it.

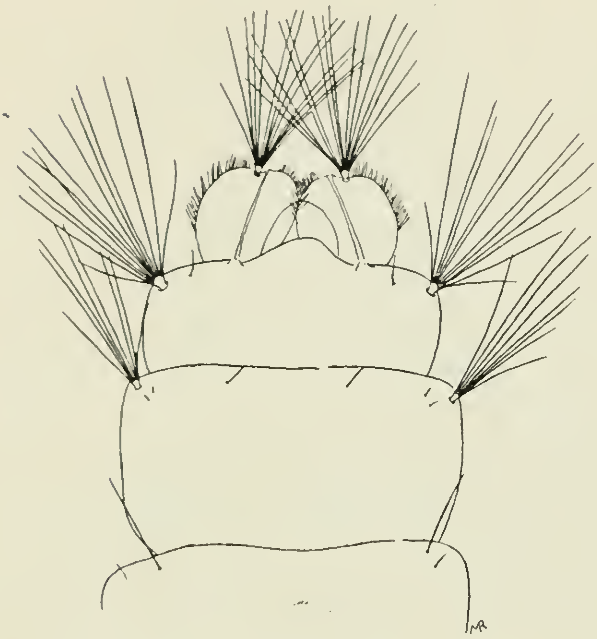

Fig. 11.

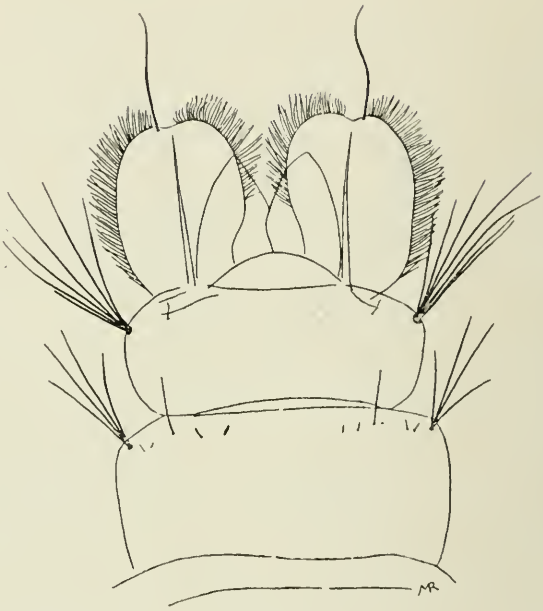

Fig. 12.

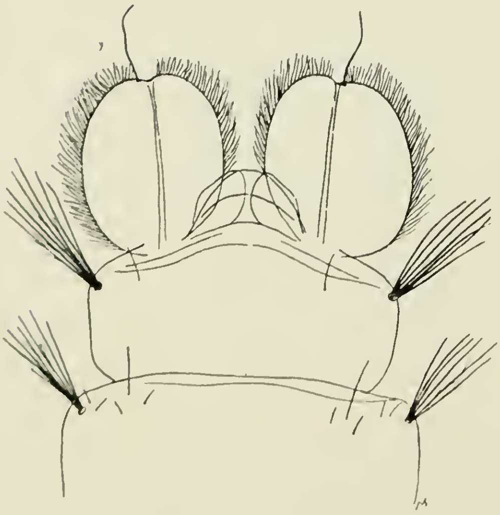

Fig. 13.

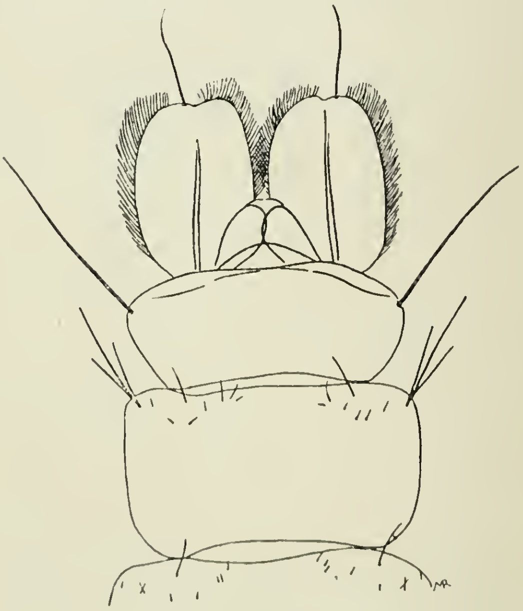

Fig. 14.

Uranotænia ornata (Fig. 15) was the only species of the genus taken; it has quite distinctive paddle characters.

Culex tigripes (Fig. 16) has broad fringeless paddles, with two small fan-branched lateral setæ on the eighth segment.

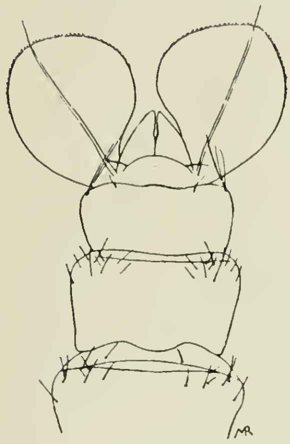

Fig. 15.

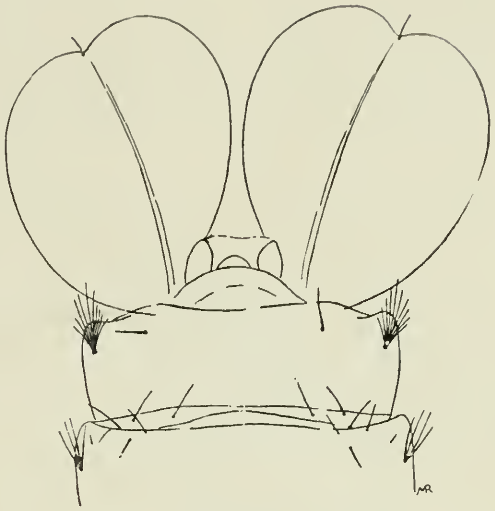

Fig. 16.

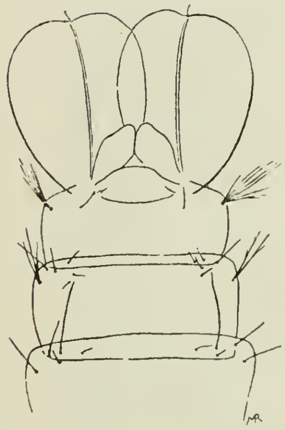

Fig. 17.

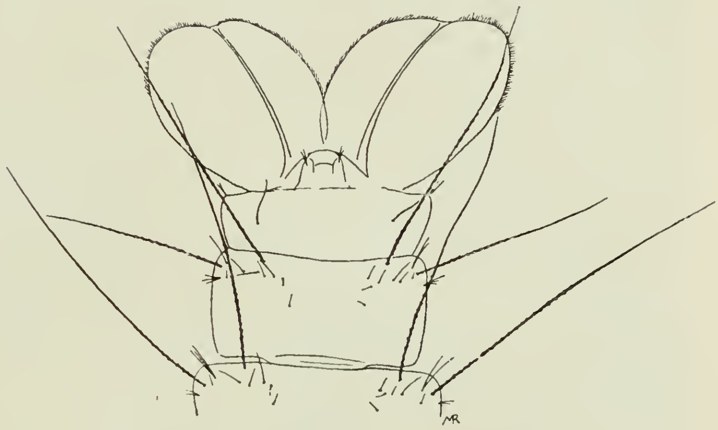

Fig. 18.

Culiciomyia nebulosa (Fig. 17) has much the same general type of paddles, but differs as regards these lateral setæ.

Toxorhynchites brevipalpis (Fig. 18).—The paddles of this giant species have no terminal spine; the lateral setæ on the eighth segment, which are specially developed on the other specimen figures, are small and inconspicuous, but certain dorsal setæ on the seventh and eighth abdominal segments are very long and markedly serrated.

Anopheles.—The only two species obtained were *Pyretophorus costalis* (Fig. 19) and *Myzomia funestus* (Fig. 20). They are almost identical in the character of the terminal segments and paddles, the only noticeable point of difference being that the lateral setæ on the eighth abdominal segment of *funestus* are larger, with less spread of the branches than in *costalis.* This difference is, however, rather more accentuated in the illustration than it should be.

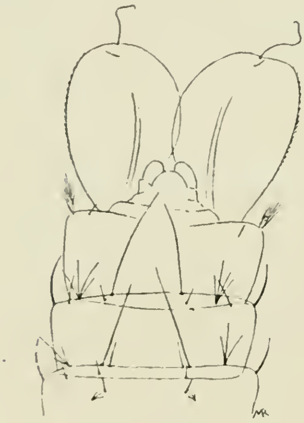

FIG. 19.

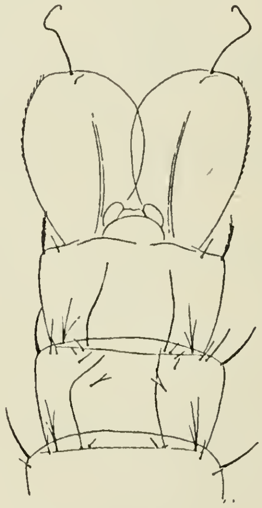

FIG. 20.

VIII.—NOTE REGARDING THE REARING OF SEVERAL SPECIES FROM DEAD LEAVES TAKEN FROM AN EMPTY WATER-HOLE DURING THE DRY SEASON.

In the early part of January a small number of fallen leaves in a dry water-hole at the root of a cottonwood tree were collected and examined for eggs (*see* Plates 1 and 2, p. 6). About thirty leaves were carefully examined under a binocular dissecting microscope. Eggs were found on several leaves; examples were sent home to be photographed by Mr. F. Noad Clark, and for exhibition at one of the meetings of the Yellow Fever Commission; these last were passed on to Mr. Malcolm Evan MacGregor, of the Wellcome Bureau of Scientific Research, who reared a number of specimens of *Stegomyia fasciata* from the eggs.* Specimens of *S. fasciata* were also reared in Freetown from eggs laid on one of the leaves.

PHOTOGRAPH OF EGGS OF *STEGOMYIA FASCIATA.*

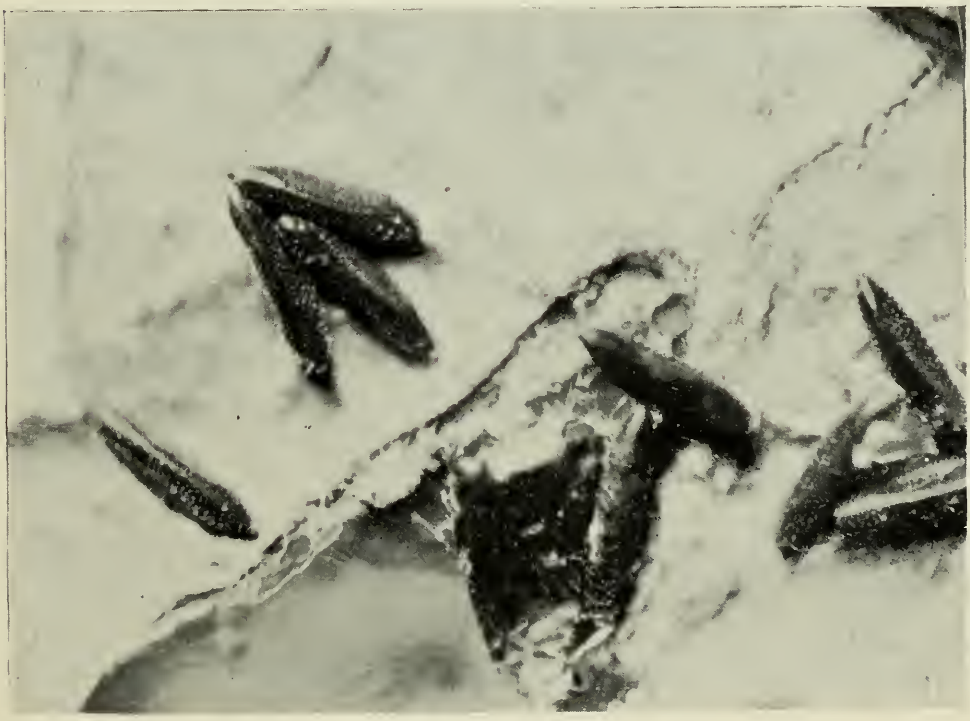

Photo by F. Noad Clark.] *Plate No.* 23.

Eggs of *Stegomyia fasciata*, laid on a leaf of Cottonwood tree. ×40.

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* *Journal of Tropical Medicine and Hygiene.* "Notes on the rearing of *Stegomyia fasciata* in London," Vol. XVIII., No. 17, pp. 193–196.

During the latter part of January a further collection of fallen leaves was made from the dry water-holes* in the same tree, but, as time did not permit of their examination for eggs, they were left lying in a paper envelope on the laboratory bench. No opportunity for careful search having occurred by the 13th March, the leaves were placed in a new, carefully cleaned petroleum tin and about 5,000 cc. of tap water added. By the 15th March the water was swarming with larvæ of all sizes up to the third skin. A few were examined and found to be larvæ of *S. fasciata.* A number of dead cockroaches were placed in the tin on this date. On the 17th March (four days after the immersion of the leaves) about 30 pupæ were captured and transferred to glass jars. On the 18th a further 50 were caught in the tin ; 9 males of *S. fasciata* emerged from the first batch of pupæ one-hundred-and-twenty-six hours after the leaves were placed in water. On the 19th, 37 more males and 4 females of *S. fasciata* emerged ; over 100 specimens of both sexes emerged on the 20th March, and 1 male specimen of *S. simpsoni*; on the 21st March, in addition to *S. fasciata*, more specimens of *S. simpsoni* and a number of *S. luteocephala* and *Ochlerotatus apicoannulatus* emerged. There was rather a heavy death rate among the larvæ in the tin, due apparently to too large a number of cockroaches being added and causing a greasy scum.

The total numbers reared were :—

	S fasciata.		*S. simpsoni.*		*S. luteocephala.*		*O. apicoannulatus.*	
	Males.	Females.	Males.	Females.	Males.	Females.	Males.	Females.
March 18 ...	9	...	...	...	...	...	...	...
,, 19 ...	37	4	...	...	...	...	...	...
,, 20 ...	25	72	1	...	...	...	...	...
,, 21 ...	19	57	7	8	19	1	5	6
,, 22 ...	4	4	...	6	24	7	...	3
,, 23 ...	1	1	...	2	3	25	...	...
,, 24 ...	...	...	...	...	...	2	...	...
	95	138	8	16	46	35	5	9

---

* Although there was no water in the holes at this date, the matted mass of decaying leaves at the bottom was still moist ; these were not disturbed, only the loose undecayed leaves being collected.

From this record it will be seen that *S. fasciata* is easily the quickest breeder, the average times being: *S. fasciata*, 7·1 days; *S. simpsoni*, 8·4; *S. luteocephala*, 9·1; and *O. apicoannulatus*, 8·2. Perhaps the chief point of importance in this breeding record is the proof it affords of the passage through the dry season in the egg state of *S. simpsoni*, *S. luteocephala*, and *Ochlerotatus apicoannulatus*.

A careful watch was kept on the breeding tin for larvæ that were expected to emerge from resistant eggs, but none occurred, although cooling was resorted to. It is a possible, if not a very satisfactory, explanation that the grease in the water from the cockroaches proved fatal to the late hatched larvæ.

IX.—METEOROLOGICAL CONDITIONS AS AFFECTING THE BREEDING AND DISPERSAL OF MOSQUITOES AT FREETOWN.

The character of the incoming rainy season, together with the tornadoes which herald its approach, is probably of great importance in this connection, as quite small divergencies may cause exaggerated effects in regard to the numbers and distribution of the various species.

The early tornadoes (which commenced on the 7th March) were frequently of the nature of dry squalls ; if accompanied by rain at all it usually fell only towards the end of the blow. Their direction was generally from the E.S.E., travelling down the estuary of the Sierra Leone River and through the gap in the hills behind Mount Aureol. They were generally accompanied by clouds of dust ; and insects, especially small ones, such as mosquitoes, would easily be caught up and carried by the wind like snowflakes, dropping to earth behind any shelter, such as the banks of the brooks, houses and streets of Freetown would afford.

Coincident with these squalls reports of mosquitoes on the shipping in the harbour occurred, also cases of fever. Specimens of *P. costalis*, *S. fasciata* and a number of *C. nebulosa* were received from Dr. Costello of the s.s. "Transmitter," which lay some 500 or 600 yards off shore.

Some of the most extensive breeding grounds (Kissy flats, *see* Plates 13, 14 and 15, pp. 14, 16) for the *Anopheles* mosquitoes lay a few miles up river S.E. of Freetown, so that an immigration of these species into Freetown during the season of these dry squalls is a possibility to be reckoned with ; more especially is this the case, as the squalls generally take place at night, or just before daybreak, when mosquitoes are likely to be on the wing.

It is therefore of importance to the mosquito section of the sanitation of Freetown that the administrative area should be enlarged so as to include these outlying districts, and attempts made to confine the surface water to definite cuts, in place of allowing it to trickle out over the large, gently-sloping areas bordering the railway (*see* Plates 9, 10 and 13, pp. 12, 13, 14).

It is also specially important that attacks on possible mosquito breeding places should be commenced at the very onset of the rains. These are necessarily few in number, and restricted in area at this period, and might be scheduled, as they will consist of pools in the beds of the deeper drainage gullies (*see* Plates 24 and 25), and hollows, empty water-holes and small dried-up ponds. Later, when the rains are heavier and the showers more frequent, all these places will be impossible as breeding situations, because the drainage gullies (natural or artificial) will form the beds of streams so torrential in character that any larvæ or pupæ will be swept into the creeks, while the larger and more permanent pools will have attracted numbers of frogs and toads intent on breeding. The available breeding places of the mosquitoes change as the season advances, the number of collections of water being enormously increased, as even the slightest

ROCK POOLS NEAR WILBERFORCE STATION.

Plate No. 24.

Rock Pools in roadside drain. During early May these pools swarmed with pupæ of *Stegomyia* ; in June and July the constant rainfall kept them washed clear.

hollows can be utilized when showers are constantly renewing the water in them (*see* Plates 4, 5 and 7, pp. 8, 9, 11). Very great risks of drying up are taken by the species, as the notes on *Stegomyia sugens* on page 132 will show, but mosquitoes, and especially *Stegomyia*, with its serial hatching eggs, are adapted to these precarious conditions; the constant dangers from predaceous enemies and competition by other organisms, whose life cycle is not so rapid, in the more permanent collections of water being of more moment to the species than the mortality arising from climatic conditions.

MOSQUITO BREEDING POOL.

Plate No. 25.

Rock Pool in the gutter of Murray Town Road, which was tenanted by swarms of pupæ of *Stegomyia sugens*.

X.—EXPERIMENTS DEALING WITH THE DESTRUCTION OF *STEGOMYIA FASCIATA.*

Experiments dealing with the destruction of *Stegomyia fasciata* were limited to the egg, larval and pupal stages, and the use of simple and easily obtained articles—ordinary burning petroleum, soft soap, flake naphthalene and salt water (from the harbour)—one of the objects of the trials being to ascertain to what extent the eggs were destroyed by methods readily applicable to the larval stage. The tests carried out with emulsions were directed chiefly against the active larval and pupal stages, the attenuated quantities of emulsions used having but little effect on the resistant eggs.

No experiments were conducted with the adult insects, as with the limited time and means at my disposal the possibility of improving on the already recorded remedies and tested methods of applying them seemed remote.

In order to render the tests as strictly comparative as possible, the trials with the different substances were carried out at the same time, the eggs, larvæ or pupæ being taken from a common source, the test on eggs with petroleum-soft soap emulsion being an exception.

Experiment No. XLIX.

COMPARATIVE EFFECT OF SOFT SOAP AND PETROLEUM (1 IN 600) ON EGGS, LARVÆ AND PUPÆ.

300 cc. of tap water was placed into each of two large beakers of three-and-a-half inches diameter.

A portion of a large batch of eggs of *Stegomyia fasciata* recently laid on filter paper was then submerged in each of the beakers, and a number of active, well-grown larvæ and pupæ were also put in.

·5 cc. of petroleum (= 1 in 600) was dropped on the surface of the water in beaker *P.*, while ·5 gram of soft soap (= 1 in 600) was dropped into beaker *S.S.*

50 eggs of the same batch were placed in salt (harbour) water as a control.

Temperature of water about 80° F.

Within 1 *hour* most of the larvæ and pupæ were dead in both of the beakers.

After 20 *hours.*

Effect on eggs :—

P.—Some of the eggs have hatched and the young larvæ are swimming about.

S.S.—No young living larvæ are visible.

Salt Water Control.—A number of eggs have hatched and the young larvæ are dead.

The eggs were then removed from the water and halved, one-half being returned to the beakers and the other carefully examined under the binocular microscope :—

P.—4 eggs had hatched out of 61 examined.

S.S.—No eggs had hatched out of 68 examined.

These half batches were then dried and put into clean tap water to test the effect of 20 hours' immersion in petroleum and soft soap on them.

After 48 *hours' immersion.*

P.—Many more eggs have hatched and larvæ are living ; at least one has moulted.

Of the half removed to tap water, 28 have hatched and larvæ are living.

S.S.—Of the eggs left in the beaker, 31 out of 60 have hatched, but no living larvæ are visible, the water is too cloudy to admit of certainty as to all being dead. Of the half removed to tap water 39 have hatched, all of which are living.

Salt Water Control.—31 out of about 50 eggs put in have hatched; only one larva is living (it died shortly afterwards).

After 96 *hours.*

P.—The water still smells of petroleum, but the larvæ which hatched from the eggs are doing well.

S.S.—No signs of living larvæ.

Of the eggs removed from the beakers after 20 hours' immersion in water to which petroleum or soft soap had been added, all but two or three doubtfully fertile eggs have hatched.

In the salt water control 8 more larvæ have hatched and the larvæ are dead.

After 8 *days.*

P.—A large number of small, underfed but active larvæ are present. All the eggs have hatched.

S.S.—No living larvæ. All the dead ones found are in the first skin. A few unhatched eggs present. After drying and re-immersing these resistant eggs in clean water 13 hatched within two days, but about half the larvæ died shortly after emerging.

CONCLUSION.

Petroleum.—1 in 600 kills all larvæ and pupæ but fails to destroy the eggs, and the larvæ which hatch survive.

Soft Soap.—1 in 600 kills all larvæ and pupæ and also the young larvæ which hatch out from submerged eggs. It has no effect on eggs which have been submerged for 20 hours, but has detrimental effects on a proportion of those which have been submerged for 8 days.

Experiment No. L.

Second Trial.

CONTRASTED EFFECT OF PETROLEUM (1 IN 4,000), SOFT SOAP (1 IN 8,000), AND NAPHTHALENE (1 IN 8,000) ON EGGS, LARVÆ AND PUPÆ OF *STEGOMYIA FASCIATA.*

In this experiment thoroughly cleaned petroleum tins cut down to six inches in depth were used. The size of the tins is nine inches by nine inches with curved corners. Approximate surface area, 80 square inches.

4,000 cc. of tap water was poured into each tin and a portion of a large batch of eggs, laid three months previously on filter paper and stored dry, was submerged in the water. A number of active larvæ and pupæ from the breeding pans were also added.

P.—1 cc. of petroleum was dropped on the water in one tin.

S.S.—·5 gram of soft soap was dropped into another.

N.—·5 gram of flake naphthalene was scattered on the water of the third.

All the pans were covered with cheese cloth. Temperature of water about 80° F.

After 24 *hours.*

P.—11 adults have emerged and are living. The larger proportion of the larvæ and pupæ are dead, but a number are still living.

Eggs.—Large numbers of eggs have hatched; some of the young larvæ are dead but the great majority are active.

S.S.—15 adults have emerged and are living. Most of the pupæ and larvæ are still active but a few are dead.

Eggs.—Swarms of newly-hatched larvæ are swimming about. No dead observed.

N.—The pupæ seem to be quite unaffected, but all the adults that have emerged are dead. All the larvæ, save a few inactive, sickly-looking ones, are dead.

Eggs.—Numbers have hatched but there are no newly-hatched living larvæ to be seen.

The larvæ which hatched out in *P.* beaker of the first test (1 in 600) (Experiment No. XLIX.) and lived for eight days were put into *N.* pan of this series.

After 48 *hours.*

P.—10 more adults out. All the remaining pupæ and most of the active larvæ which were put in are dead.

Eggs.—The larvæ which have hatched out are now very numerous. A small proportion are dead, but most are quite active and many are in their second skins.

S.S.—9 more adults out. The greater number of the larvæ put in and a few pupæ are still living.

Eggs.—Swarms of active newly-hatched larvæ are visible.

N.—The flakes of naphthalene have now largely disappeared from the surface of the water; some have sunk, others have doubtless evaporated.

21 more adults are out—11 dead, 10 living. Only 3 living larvæ, of those originally put in and those transferred from the *P.* beakers in the first trial, remain.

Eggs.—Large numbers of dead newly-hatched larvæ are visible but no living ones.

CONCLUSION AT 48 HOURS.

Petroleum.—1 in 4,000 (1 cc. to 80 square inches area), while generally effective as a larvicide, allowed of a few survivors. It is very ineffective as against pupæ, a considerable proportion being able to complete their development and successfully emerge as adults. Its effect on submerged eggs or the larvæ hatching from them is negligible.

Soft Soap.—1 in 8,000 is practically useless. It kills only a small proportion of the active larvæ and pupæ and has no effect on the eggs or young larvæ that emerge from them.

Naphthalene.—1 in 8,000 is not effective as against pupæ, but (in a covered pan) kills all adults that emerged within 24 hours and about 50 per cent. of those emerging on the second day. With a few exceptions it is fatal to all larvæ, those emerging from eggs as well as those present from the start, for a period of 48 hours.

Experiment No. LI.

Sequel to Experiment No. L.

After 8 *days.*

P.—The few resistant larvæ of those put in continued to live, while the young larvæ that hatched from the eggs grew and thrived so far as a rather short allowance of food would allow them.

·5 gram of flake naphthalene was sprinkled over the surface of the water and the cheese cloth cover removed. Within 24 hours the great majority were either dead or dying.

A few survivors were still living after 48 hours, when a further gram of flake naphthalene was sprinkled on the water, the pan remaining uncovered.

Although the numbers living when this additional quantity of naphthalene was added were reduced a few of the larvæ survived and pupated.

S.S.—Upon the conclusion of the 48 hours' test there were a considerable number of pupæ and well grown larvæ present in addition to the swarms of young ones that had emerged from the eggs. Food (dead grasshoppers) was placed in the tin and another ·5 gram of soft soap added, bringing soap content up to 1 in 4,000. This addition had no apparent effect in reducing the numbers after 48 hours; the young larvæ had grown and some of the older ones had pupated.

The addition of another gram brought the soap content up to 1 in 2,000. 72 hours later observation showed that the increase was partially effectual. Adults emerged from the pupæ and some of the better grown larvæ were still active, but the great proportion of the young larvæ were dead, although a few which had reached the third skin survived.

A third gram of soft soap was added (3 in 4,000). This also proved after 48 hours to be only partially effectual, living larvæ and pupæ being still present. 24 hours after the addition of a fourth gram (1 in 1,000 strength) all the survivors were dead. A fresh batch of larvæ and pupæ were added, and these also were all killed within 24 hours.

Of a further batch which were placed in the pan some survived until the second day, but all were dead after 72 hours.

Naphthalene.—In addition to the trial with the surviving larvæ of *P.*, in Experiment No. L., as above recounted, a few of the older larvæ which had survived the 48 hours' trial at 1 in 8,000 naphthalene were living, together with a number of young ones which had hatched out a day or two later when the effects of the naphthalene had passed off. A gram of naphthalene was sprinkled on the surface of the water to try the effect of 1 in 4,000 without the cover to the pan; most of the young larvæ were quickly killed off, but a few survived.

With a covered pan 1 in 4,000, however, proved effective, all the larvæ and pupæ or the adults emerging from them being killed.

CONCLUSION ARISING FROM THE SEQUEL.

Soft Soap.—At less strength than 1 in a 1,000 soft soap is not an effective larvicide, but at this concentration it is decidedly superior to petroleum and is more lasting in its effects. Its effectiveness, however, gradually ebbs.

Naphthalene.—At 1 in 4,000 (1 gram to 80 square inches) kills all larvæ and pupæ or the adults emerging from pupæ in a pan with a cheese cloth cover. It is not, however, totally effective if the cover is removed.

Experiment No. LII.

Third Trial.

EFFECTIVENESS OF PETROLEUM AND SOFT SOAP, AND PETROLEUM, SOFT SOAP AND NAPHTHALENE IN EMULSIFIED FORM AS COMPARED WITH PETROLEUM ALONE TO DESTROY LARVÆ AND PUPÆ.

The emulsions used were constituted as follows:—*Petroleum and soft soap.* 3 parts soft soap melted by heat in 15 parts of water; 100 parts of petroleum added very gradually with much stirring and shaking while the soft soap was still hot.

Petroleum, Soft Soap and Naphthalene.—4 parts soft soap melted in 20 parts of water. 15 grams of naphthalene was gradually added to 100 cc. of petroleum (this appears to be the saturation limit at 80° F.). The petroleum with the naphthalene in solution was then added very gradually, with much stirring and shaking, to the hot soft soap solution.

In practice it was found best to dilute the small quantities of emulsions used with a little water before adding it to the pans which contained the larvæ and pupæ.

Tests.—These were conducted in cleaned empty petroleum tins cut down to six inches in depth; the surface area was approximately 80 square inches and the water contents 4,000 cc. Tins with cheese cloth covers.

Period 20 *hours.*

(1) *Petroleum.*—1 in 4,000 killed 7 pupæ and 37 larvæ. 1 pupa survived and an adult emerged but died.

P. S.S. emulsion.—1 in 8,000 killed all, 11 pupæ and 31 larvæ.

P., N. and S.S. emulsion.—1 in 8,000 killed all, 14 pupæ and 47 larvæ.

(2) *Petroleum.*—1 in 8,000.

After 20 hours 3 adults emerged and died, 5 pupæ and 3 larvæ living, 40 larvæ dead.

After 48 hours 3 more adults emerged, 1 living, 2 dead; 1 pupa living, 1 pupa and 3 larvæ dead.

P. S.S. emulsion.—1 in 16,000.

After 20 hours 1 adult emerged and died; 8 pupæ dead; 62 larvæ dead; 1 larva just capable of movement, but unable to leave the surface; was dead next morning.

P., N. and S.S. emulsion.– 1 in 16,000.

After 20 hours 10 pupæ dead; 36 larvæ dead; 1 larva just capable of movement, but unable to leave the surface; was dead next morning.

(3) *P. S.S. emulsion.*—1 in 20,000.

After 20 hours 23 pupæ dead; 27 larvæ dead; 3 pupæ and 5 larvæ swim freely; 2 larvæ make feeble movement, but cannot leave the surface.

After 40 hours 5 pupæ and 1 larva survive and are active.

P., N. and S.S. emulsion.—1 in 20,000.

After 20 hours 3 adults emerged and died; 20 pupæ and 29 larvæ dead; 1 pupa living; 4 larvæ make feeble movement, but cannot leave the surface.

After 40 hours all the survivors dead.

A second trial with *P., N. and S.S.* at 1 in 8,000 killed all the larvæ and pupæ put in the same day, and rendered a second batch put into the same tin on the following day incapable of active movement within one hour, but two or three of this second batch of larvæ survived until the next day.

CONCLUSION.

Petroleum, soft soap and naphthalene when blended together in emulsified form are much more effective and economical in use than when used separately.

P. and S.S. emulsion killed all at 1 in 16,000 and a large proportion at 1 in 20,000.

P., N. and S.S. emulsion killed all at 1 in 20,000.

Experiment No. LIII.

EFFECT OF PETROLEUM AND SOFT SOAP EMULSION ON THE EGGS OF *STEGOMYIA FASCIATA.*

This test was not carried out at the same time as the larval and pupal trials, but several months later.

Petroleum and soft soap emulsion used, 1 in 8,000 dilution.

Eggs laid not more than three weeks and dried about a week previously.

After 22 hours.

144 out of 156 eggs had hatched (= 92 per cent.), 2 larvæ showed feeble movement, and the rest were dead.

12 unhatched eggs transferred to tap water.

Transferred Eggs.—6 hatched next day, the larvæ lived.
Remaining eggs opened on the ninth day.
3 living larvæ and 2 dead ones extracted.
Remaining egg infertile.

Control.—A portion of the same batch of eggs, 196 in number, was placed in tap water.
81 hatched (= 41 per cent.), 1 larva died.

Recently-laid eggs that had been kept moist were also tested in a separate pan :—
36 out of 42 hatched in 22 hours (= 85 per cent.) and died.
6 unhatched eggs were transferred to tap water.
All 6 of the transferred eggs hatched next day ; the larvæ lived.

A batch of eggs that had been stored dry for 4 months was submerged in the same diluted emulsion, 1 in 8,000.

Out of 51 eggs, some of which showed signs of shrinkage, 6 hatched within 22 hours and 2 uncapped, but the still living larvæ had not emerged at the time of observation. The remaining eggs were removed to tap water. The larvæ which emerged in the emulsion all died.

After transference to Tap Water.

Next day 6 eggs uncapped :—2 larvæ out, making feeble movements.
2 ,, ,, , dead.
2 larvæ failed to get free of shells.
2nd day 2 hatched :—1 dead, 1 living.
3rd ,, 5 ,, 3 ,, 2 ,,
4th ,, 5 ,, 4 ,, 1 ,,
5th ,, 4 ,, 1 ,, 3 ,,
6th ,, 4 ,, All dead.

On the 9th day the unhatched eggs were opened. 1 living larva was extracted ; all the other eggs contained either dead larvæ or were infertile.

CONCLUSION.

Submergence in petroleum-soft soap emulsion (1 in 8,000) is not effective in killing larvæ within the eggs, though it is possibly the cause of some mortality. It is, however, effective in that it induces a high percentage of the less resistant eggs to hatch at once, when the young larvæ are killed.

XI.—EFFECT OF SALT WATER (FROM FREETOWN HARBOUR) ON THE EGGS, LARVÆ AND PUPÆ OF *STEGOMYIA FASCIATA.*

In their conception these experiments were complementary to those performed by Macfie (1913–14) with common salt (“A Note on the action of Common Salt Water on the Larvæ *Stegomyia fasciata*,” “Bulletin of Entomological Research,” Vol. IV., Pt. 4, pages 339–344, by Dr. J. W. Scott Macfie, M.A., M.B., Ch.B., West African Medical Staff), their purpose being to test the effect of salt water on the eggs, a point Dr. Macfie did not deal with.

Experiment No. LIV.

EFFECT ON EGGS, LARVÆ AND PUPÆ CONTRASTED.

Preliminary.—A large batch of several hundred eggs laid two months previously was divided; half were put into water taken from the harbour and half into tap water.

A number of active larvæ and pupæ from the breeding pans were also put into the salt water. Temperature 80° F.

The first eggs to hatch were some of those in the salt water (within 15 minutes of immersion), those in the tap water commenced a few minutes later. None of the larvæ which hatched from the eggs placed in salt water survived more than an hour.

After 16 hours.—All the active larvæ placed in the salt water were dead. From the pupæ many adults had emerged and were living; the remaining pupæ were all living and successfully completed their development.

Eggs.—Two or three hundred had hatched, the number of larvæ which had emerged being larger in the salt than in the tap water. In the salt water all were dead, in the tap water all were living. After 48 hours a careful examination showed that of the eggs placed in salt water all had hatched save a few, which subsequent dissection showed contained dead larvæ.

Of the portion of the batch of eggs placed in tap water many were unhatched. Larvæ continued to hatch from the eggs at intervals.

Experiment No. LV.

TRIALS WITH EGGS ONLY.

Salt water from the harbour was filtered and 170 cc. placed into each of two beakers, *D* and *M*.

Controls.—170 cc. of tap water was placed in each of two beakers *Dc.* and *Mc.*

Eggs dry: laid on filter paper a month previously and stored dry. The slip of paper was divided and one portion with 102 eggs on it placed in *D.*, the other, with 106 eggs on it, placed in *Dc.*

Eggs kept moist; laid a month previously and stored under conditions which kept the filter paper on which they were laid moist.

The slip of paper was divided, a portion with 107 eggs on it was placed in *M* and the other with 95 on it was placed in *Mc.*

D. 102 dry.—After 2 hours' immersion 3 had hatched; larvæ dead.
,, 22 ,, ,, 89 ,, ,, ; ,, ,,

Microscopic examination showed that 95 eggs had uncapped, but 2 larvæ died without emerging. Of the 7 remaining eggs 2 collapsed on drying, 5 retained their shape. These 5 eggs resisted continued immersion in tap water for 20 days, during which period they were subjected to "cooling" on five occasions. Dissection showed that 4 of the eggs contained fully developed larvæ, 2 of which were living, 1 showed no movement and 1 showed signs of decay.

Total hatched, 89 = 87 per cent.

Dc. Tap water control.—106 eggs (a portion of the dry batch used in *D*).
After 2 hours' immersion 33 had hatched and were living.
,, 22 ,, ,, 1 ,, ,, ,, ,, ,,

Microscopic examination showed that 34 eggs had uncapped. Of the 72 remaining eggs 2 had collapsed while immersed and 2 more collapsed on drying. 68 were re-immersed in tap water.

After 1 day	2	hatched.
,, 3 days	1	,,
,, 4 ,,	1	,,
,, 8 ,,	4	,,
,, 9 ,,	11	,,
,, 10 ,,	18	,,
,, 11 ,,	4	,,
,, 16 ,,	3	,,
,, 18 ,,	2	,,
	46	

50 per cent. of salt water was then added to the tap water in the pan.
The same day 1 hatched larva lived.
After 6 days 2 ,, larvæ ,,
,, 18 ,, 3 ,, ,, ,,

All the remaining eggs were dissected and 5 living larvæ were extracted, the remaining 11 eggs contained dead larvæ or were infertile. Total hatched, 81 per cent.

M. 107 Moist.—After 2 hours' immersion 49 had hatched—all dead.
,, 22 ,, ,, 46 ,, ,, ,,

Microscopic examination showed that 98 had uncapped; 3 larvæ had died without emerging. 5 eggs had collapsed while in the water. 1 collapsed on drying. 3 retained their shape.

These 4 eggs were then immersed in tap water.
After 1 day 1 hatched, and the larva lived.
,, 9 days 1 ,, ,, ,, ,, ,,
,, 10 ,, 1 ,, ,, ,, ,, ,,

Dissection showed that the collapsed egg was infertile. Total hatched, 98 = 92 per cent.

Mc. Tap water Control.—95 eggs (a portion of the moist batch used in *M*).
After 2 hours' immersion 25 had hatched living.
,, 22 ,, ,, 2 ,, ,, ,,

Microscopic examination showed that 27 had uncapped, 8 eggs had collapsed while in water, 2 collapsed on drying, 58 retained their shape and were re-immersed in tap water.

After 1 day... ... ... 2 hatched living.
,, 8 days ... ... 3 ,, ,,
,, 9 ,, ... 2 ,, ,,
,, 16 ,, ... 5 ,, ,,
,, 17 ,, ... 2 ,, ,,
14

100 per cent. of salt water was then added to the tap water in the pan.
The same day 1 hatched and died.
After 11 days 1 ,, ,, ,,
,, 12 ,, 11 ,, ,, ,,

All the remaining eggs were dissected and found to contain dead larvæ or in a few instances were infertile. Total hatched, 54 = 57 per cent.

All the eggs were cooled on the 7th, 8th, 9th, 15th and 17th days after immersion.

Experiment No. LVI.

TRIALS WITH SPECIALLY RESISTANT EGGS

A large batch of eggs, which had been laid on filter paper and stored dry for two months, was immersed in tap water for five days. During this period several hundreds hatched. From among the resistant unhatched eggs two lots, each of 100 full eggs, were picked out with a wet camel hair brush and placed on wet filter paper. The slips of filter paper were allowed to dry and the collapsed eggs were removed, one of the slips was caught up by a draught during this operation and a number of eggs were lost.

There remained 65 full eggs on the one paper and 91 on the other.

The slip with 91 eggs on it was submerged in salt water, *S.W.* That with 65 eggs on it was placed in tap water as a control.

S.W.—After 20 hours 33 had hatched and died.

Tap Control.—After 20 hours 1 had hatched and was living.

After 44 hours' immersion no more larvæ had hatched. The slip was removed from the salt water and placed in tap water.

The record of the two lots is as under:—

RECORD OF THE EGGS AFTER REMOVAL FROM SALT WATER ON THE 4TH APRIL.

Lot.	Date of hatching.																
	April.														May.		
	*10	*11	12	*13	14	15	16	*18	19	*20	*23	*25	28	30	4	10	12
S.W. ... ... (In tap water after 44 hours' immersion in salt water.)	3	1	31	...	...	...	...	...	...	...	...	...	1	...	1	1	1
Tap Water Control ...	6	6	..	18	1	2	2	3	2	3	4	7	...	2	1	...	1

On the dates marked with an asterisk the pans were cooled in an ice chest. During May the pans were placed out of doors at night; the 3rd and 9th were cool nights.

The larvæ of the *S.W.* lot which hatched out on the 4th and 10th of May died after emerging.

On the 13th May the remaining unhatched eggs were dissected. 9 living larvæ were extracted from the *S.W.* lot and 5 living larvæ from the control lot. The remaining eggs contained dead larvæ or were infertile.

SUMMARY OF RESULT.

Of 91 full resistant eggs 33 (=36 per cent.) hatched in response to 20 hours' immersion in salt water (Harbour water), while only 1 (=2 per cent.) of the 65 similar control eggs placed in tap water hatched during the same period.

Subsequently, of the balance of the 91 eggs, on removal after 44 hours' immersion in salt water to tap water, 39 (=43 per cent.), hatched, all but two of the larvæ living, and 9 living larvæ were extracted from the eggs 39 days after their removal from salt water.

Mortality, 10 = 11 per cent.

Of the control eggs, 1 only had hatched during the first 44 hours' immersion. Subsequently, 58 (= 89 per cent.), hatched over a period of 39 days mainly in response to cooling, and 5 living larvæ were extracted from the egg.

Mortality, 1 = 1 per cent.

Conclusions.

The effectiveness of salt water as compared with fresh in causing eggs to hatch and killing the young larvæ is clearly demonstrated by these experiments.

The last experiment, dealing with resistant eggs, greatly modifies, however, one's views as to the practical usefulness of salt water as an insecticide for the destruction of eggs. For it is evident that though it causes the immediate hatching and destruction of recently laid eggs, a considerable proportion of resistant eggs that have been dry for a long time are not affected within a period of 44 hours, and that the larvæ contained in such eggs are not killed.

For coast towns, however, salt water, if the expense of pumping were not too heavy, might serve as a very valuable flushing agent for gutters, sewers and cess pits after clearing. It would, I am informed, probably kill any *Ankylostome* eggs with which it came in contact, and if used for flushing drainage ditches in streets and watering roads, would, in addition to its services in reducing the numbers of mosquito and *Ankylostome* eggs effectually prevent the growth of weeds on road side spaces. In Freetown the clearing and weeding such spaces must be a considerable annual cost.

In the event of the use of salt water for such purposes, it should be applied lavishly, in the light of the foregoing experiments, at the *close of the rains* if its full effectiveness for the destruction of *Stegomyia* eggs is to be obtained, because a large proportion of the eggs laid in gutters and pools, in culverts, etc., will not attain their full powers of resistance (resting capabilities) until the establishment of the dry season has caused the evaporation of water in moist places and dried the eggs.

Experiment No. LVII.

TRIAL TO ASCERTAIN IF THE STIMULUS TO HATCH EXERTED BY SALT WATER ON THE EGGS OF *STEGOMYIA FASCIATA* IS DUE TO ITS SPECIFIC GRAVITY OR SOME OTHER CAUSE.

In the absence of other means the specific gravity of filtered sea water from the Harbour was tested with a urinometer and found to be 25°. Loaf sugar was then added to tap water until the solution showed the same specific gravity.

200 cc. of the salt water, 200 cc. of the sugar solution, and 200 cc. of the tap water were placed in respective beakers referred to as "Salt," "Sugar" and "Tap" in the following record.

Two batches of eggs were used: (1) laid on filter paper and stored *dry* for 3 months; (2) eggs that had been laid on filter paper about 3 weeks and had been kept *dry* for 10 days. A portion of both batches of eggs was placed in each of the three beakers. The temperature of the fluids just prior to the immersion of the eggs was 85° F.

The number of eggs put into the beakers was :—

"Salt," 20 recent.	"Sugar," 28 recent.	"Tap," 20 recent.
291 old.	322 old.	324 old.
311	350	344

RESULT.

Lot.	Number of days after immersion that hatching occurred.											Result of dissection of unhatched eggs after 24 days' immersion.
	1	2	3	4	5	6	10	12	16	18	22	
"Salt,"												
Living ...	...	...	...	...	...	...	...	...	...	...	...	All the larvæ in unhatched eggs were dead.
Dead ...	155	19	65	9	1	...	...	...	...	1	...	
Total ...	155	19	65	9	1	...	...	...	...	1	...	250 out of 311. Mortality in egg stage, 19 per cent.
"Sugar."												
Living ...	16	52	184	17	1	...	1	...	...	...	...	All the larvæ in unhatched eggs were dead.
Dead ...	8	2	...	1	2	...	...	...	...	...	...	
Total ...	24	54	184	18	3	...	1	...	...	...	...	284 out of 350. Mortality in egg stage, 19 per cent.
"Tap."												
Living ...	138	2	18	...	18	4	6	1	1	3	25	From unhatched eggs— Recent, 2 living larvæ extracted. Old, 33 ,, ,, ,,
Dead ...	6	2	...	2	...	...	...	...	...	...	...	
Total ...	144	4	18	2	18	4	6	1	1	3	25	266 hatched. 35 extracted. 301 out of 344. Mortality in egg stage, 12 per cent.

All the pans containing the eggs were cooled in the ice chest on the 5th, 6th, 10th, 16th and 22nd days.

This result suggests that the efficacy of salt water in inducing the eggs to hatch sooner than they would in fresh water is not merely a question of specific gravity; if reference be made to the tests of the effect of petroleum-soft soap emulsion, Experiment No. LIII. 1 in 8,000 dilution, on the eggs of *S. fasciata* (page 159), we find much the same phenomena occurring. It seems probable that the action is likely to be related to the penetrating properties of the fluids used, possibly the ease of absorption by the (colloid) substance of which the bosses are formed is the crucial factor.

Immersion in the salt and sugar solution seems to have been responsible for an increase of mortality to the eggs, apart from that to the larvæ after hatching, of from 12 per cent. to 19 per cent.—the numbers involved being too large to admit of the explanation of the 7 per cent. increase in both cases being due to chance.

XII.—NOTE RESPECTING THE SIZE OF MESH FOR NETTING REQUIRED FOR USE AGAINST *STEGOMYIA FASCIATA.*

The wire gauze used for the mosquito house had a mesh of 16 × 16 to the inch; for the cages 12 × 12 to the inch mesh was used; and for the tubes, used to test the length of life of the adults, the mesh was 14 × 14 to the inch. A supply of large and small mesh cotton netting was also required to be used for repairs, covers to breeding pans, temporary cages, sleeves, etc. No definite size of mesh had been specified. Of the two sizes received one had a mesh of 10 × 13 to the inch, and the other 11 × 15 to the inch.

During the progress of the research it was found that a few well-grown (medium to large-sized) specimens of *S. fasciata* and also a number of *S. sugens* escaped from large cages, where they were well fed, through the 12 × 12 inch mesh. From the tubes, where they were crowded, and, presumably, more anxious to escape, large sized specimens, which had only recently emerged, got out in considerable numbers through 14 × 14 to the inch mesh. In order to carry out the tests for which these tubes had been specially constructed, it was found necessary to remake them with 18 × 18 to the inch mesh.

With cotton netting it was found that the mosquitoes, bred in pans covered with that having the smaller sized mesh of 11 × 15 to the inch, easily got out, presumably shortly after emergence, before their wings and limbs were fully dry; while wild specimens gained entrance through the coarser netting, having 10 × 13 mesh to the inch, and oviposited in some of the experimental pans.

No instances of escape from the mosquito house through the 16 × 16 to the inch mesh were discovered, and, as the mosquitoes in it were reared from larvæ that had ample food supplies and consequently were large sized specimens, it is probable that few, if any, escaped. Even for small specimens escape would have been difficult, owing to the fact that large numbers of spiders, both within and without, spun webs and trailed their threads over the wire gauze, so that, had this been of an even larger mesh, it would soon have been rendered mosquito-tight.

In view of the fact that, under starved conditions, specimens of *S. fasciata* are reared of little more than half the size of the normal insects, the advice of the authorities concerned with the mosquito work in the Panama Canal zone[*] that a mesh not larger than 18 x 18 to the inch should be used in relation to Yellow Fever screening, is fully justified and cannot be too strongly urged upon our own authorities.

It may be of interest to record that I was unable to get from any of the stores in Freetown or from the Public Works Department, a supply of netting of less than 12 x 16 to the inch, though I was informed by one of the stores that complete made-up nets could be supplied in the smaller mesh.[†] Subsequently, the Hospital authorities found some spare 20 x 20 to the inch netting which they had in store.

If by any means traders could be induced to stock nothing of of coarser mesh than 18 x 18 to the inch, it would be to the general welfare of the inhabitants in situations where any danger of Yellow Fever epidemics exists. The supplying of larger mesh netting, even for the purpose of repairs, is to be deprecated.

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[*] See "Monograph of the Mosquitoes of N. America, etc.," published by the Carnegie Institute, Vol. I., pages 363 and 364.

[†] Many of the nets I saw in actual use, including those at the Government rest house, were, however, of larger mesh.

XIII.—APPARATUS AND METHODS EMPLOYED.

Mosquito House. Plate 21, *page* 96.

This was designed by myself at the suggestion of Lt.-Col. Sir Ronald Ross. The woodwork was of unpainted teak, the stiffening framework of galvanized iron, and the wire gauze for the panels of phosphor-bronze. After eleven months' exposure to the weather at Freetown it was in perfect condition (*see* Figs. 21 and 22, pp 170 and 171).

Cages.

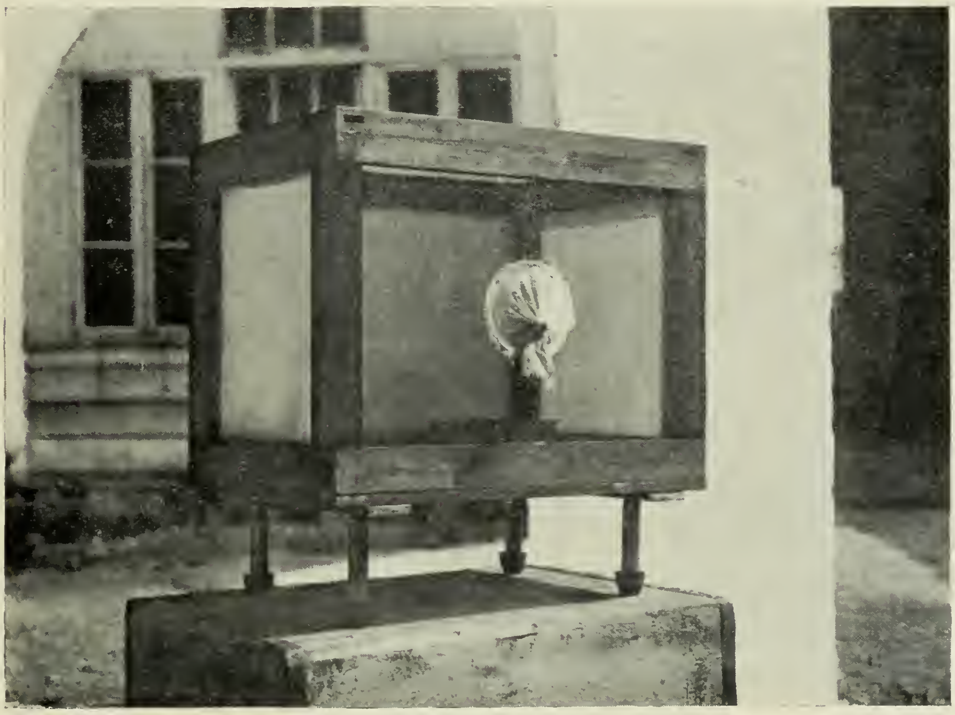
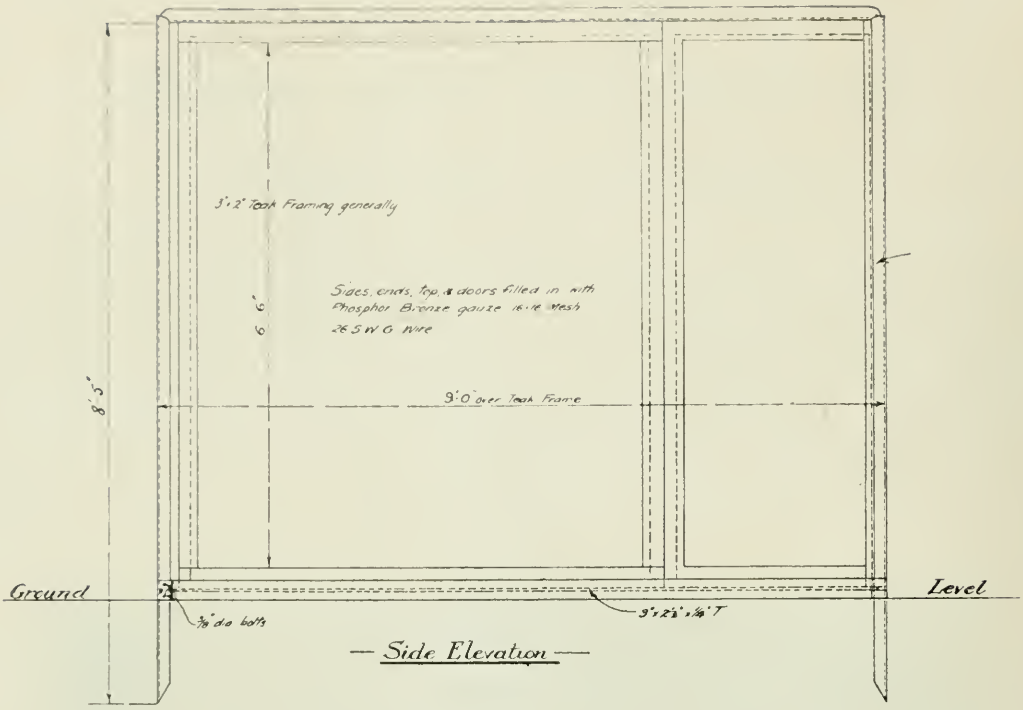


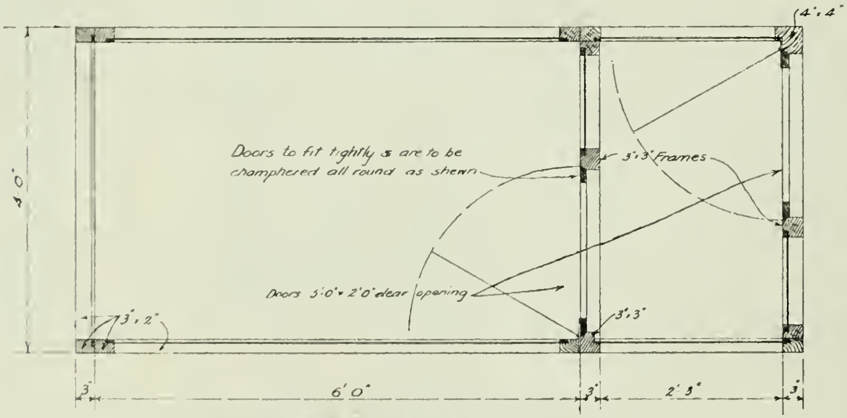
Plate No. 26.

A Mosquito Cage.

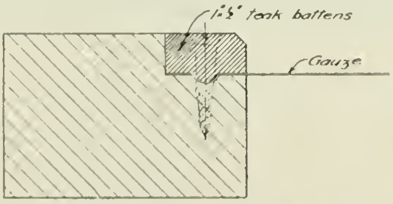
The floor boards were of teak with stiffening battens of galvanized iron, into which the legs screwed. The upper framework was of "flat oiled" pine. In the case of the cages intended for "infected" mosquitoes, the panels were of phosphor-bronze wire gauze. It was found convenient to cover this with thin cotton on the inner side.


3"×2" Teak Framing generally
Sides, ends, top, & doors filled in with Phosphor Bronze gauze 16×16 mesh 26 S W G Wire
6' 6"
8' 5"
9' 0" over Teak Frame
Ground
Level
5/8" dia bolts
9"×2½"×¼" T

— Side Elevation —


4"×4"
Doors to fit tightly & are to be champhered all round as shewn
3"×3" Frames
Doors 5'0"×2'0" clear opening
3"×3"
3"×2"
4' 0"
3"
6' 0"
3"
2' 3"
3"

— Sectional Plan (Shewing timber work) —



— Method of attaching Gauze to Framework. —

FIG. 21. PLAN OF MOSQUITO HOUSE.

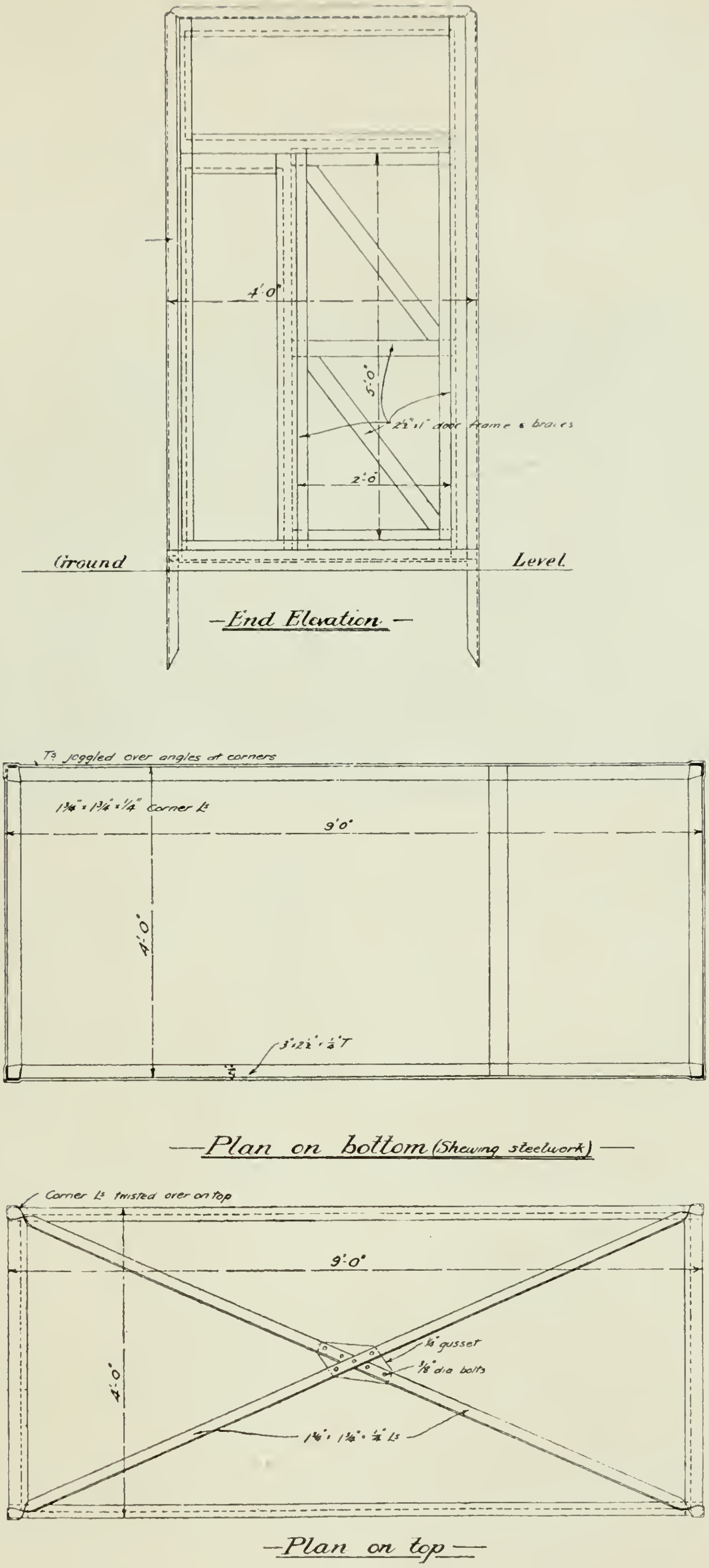


FIG. 22. PLAN OF MOSQUITO HOUSE.

For "uninfected" mosquitoes only the front panel, supporting the sleeve, was of wire gauze, the top and remaining sides being covered with cotton only. These cages, built to my own design, proved to be very serviceable and useful, and both in size and construction are suitable for a wide range of insect breeding. They can be taken down in a few minutes, and when packed flat take up a minimum of room, so that they can be easily transported.

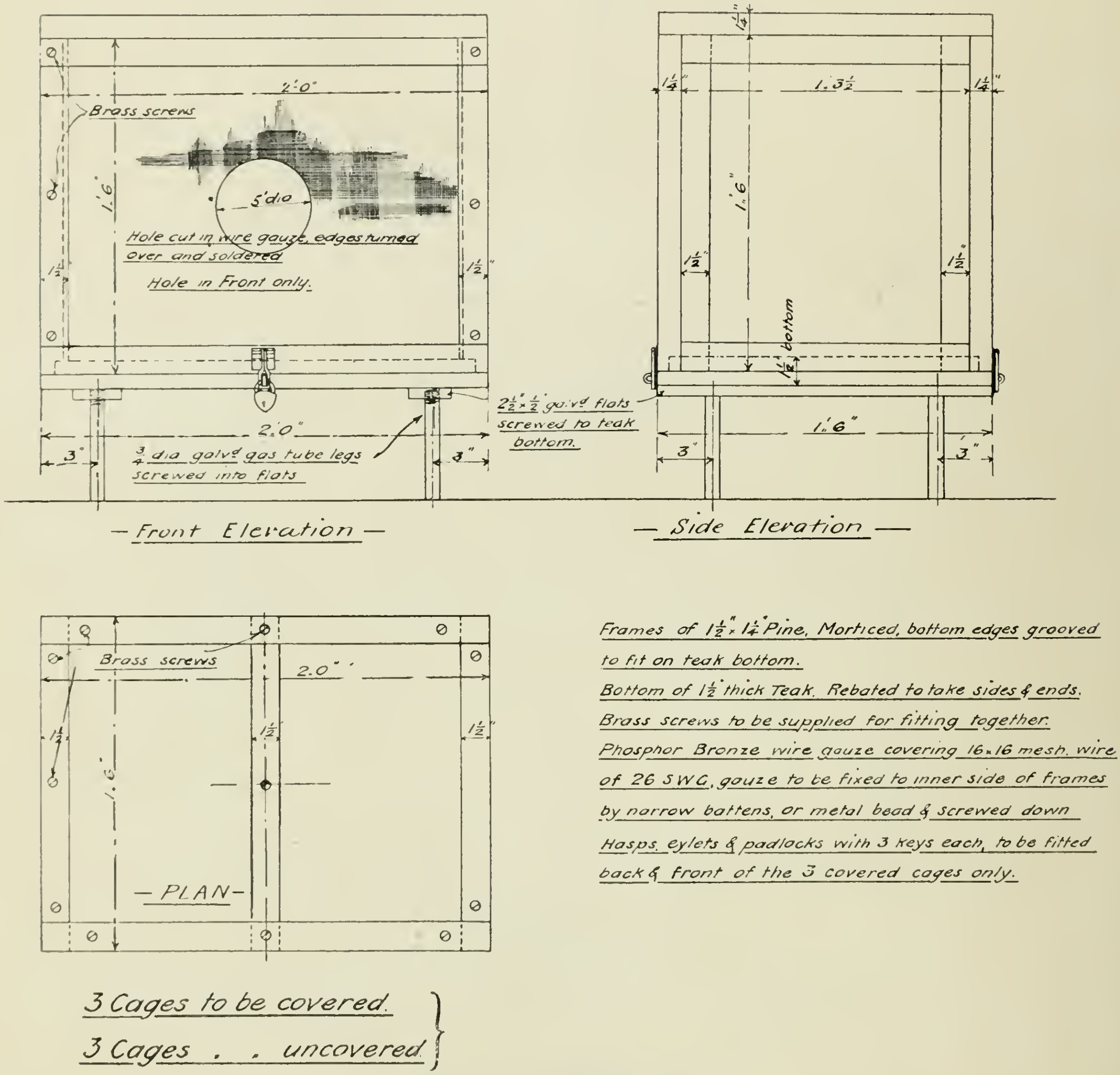

2'0"
Brass screws
5" dia
1'6"
1½"
Hole cut in wire gauze, edges turned over and soldered
Hole in Front only.
2'0"
3"
¾ dia galvᵈ gas tube legs screwed into flats
2½"×½" galvᵈ flats screwed to teak bottom.
— Front Elevation —
¼"
1'3½"
1½" bottom
1'6"
— Side Elevation —
Brass screws
2.0"
— PLAN —
Frames of 1½"×1¼" Pine, Morticed, bottom edges grooved to fit on teak bottom.
Bottom of 1½" thick Teak. Rebated to take sides & ends.
Brass screws to be supplied for fitting together.
Phosphor Bronze wire gauze covering 16×16 mesh. wire of 26 SWG, gauze to be fixed to inner side of frames by narrow battens, or metal bead & screwed down
Hasps, eylets & padlocks with 3 keys each, to be fitted back & front of the 3 covered cages only.
3 Cages to be covered.
3 Cages " " uncovered

FIG. 23. PLAN OF CAGE.

Pens.

These were of phosphor-bronze wire gauze, supported on a light galvanized iron frame. Their use was to cover stored eggs, pans of water containing larvæ, or tubes containing adults when it was desired to leave these exposed in the open air (*see* Fig. 24, page 174).

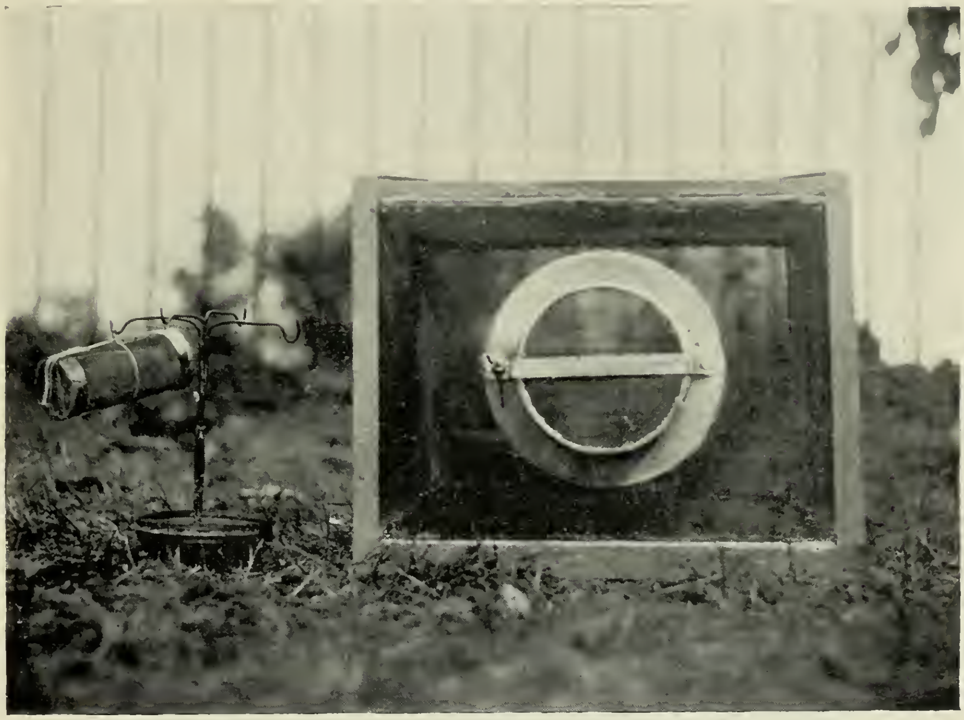


Plate No. 27.

Mosquito Pen and wire gauze tube for testing length of life of adults in various situations. Note method of suspension so as to isolate from ants.

Wire Gauze Tubes.

These were of wire gauze opening at either end (*see* Fig. 25, page 174); they were used to intern adult mosquitoes to test their length of life, and were also used to protect stored eggs from disturbance by small animals and the larger insects (*see* Plates Nos. 27 and 28).

Hurricane Lamp Chimneys.

These can be converted into useful little cages by covering the upper opening with fine gauze and putting the open end down on a pad of filter paper placed in a tin lid (*see* Plate No. 28, page 175).

Methods.

To obtain eggs.—The method mentioned by Christophers and Stephens (1908) of confining females in inverted screw-topped jars was found to answer well for single individuals. With *Stegomyia fasciata* and *S. sugens*, flooded blotting paper in the cover is sufficient to

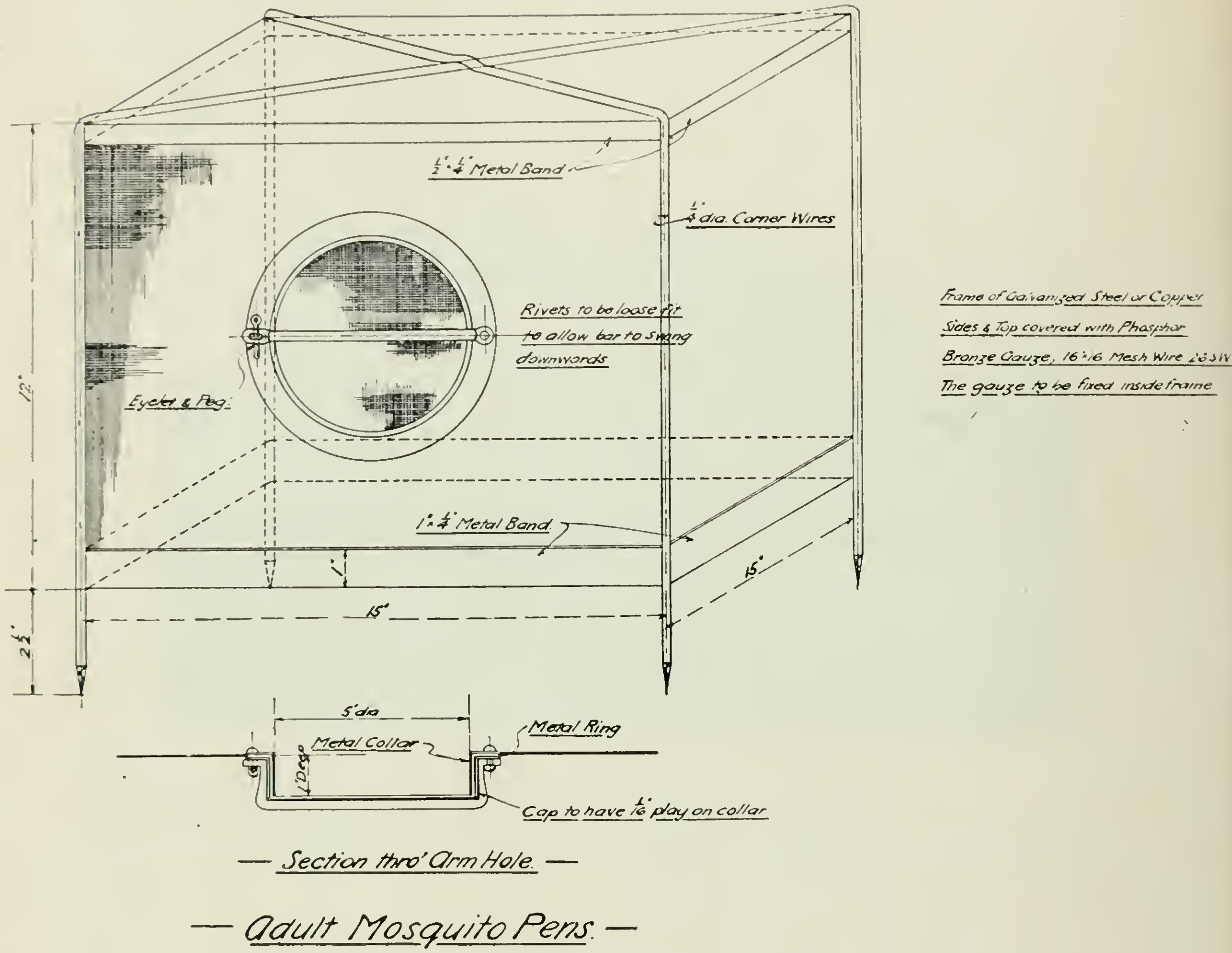


FIG. 24. PLAN OF PEN.

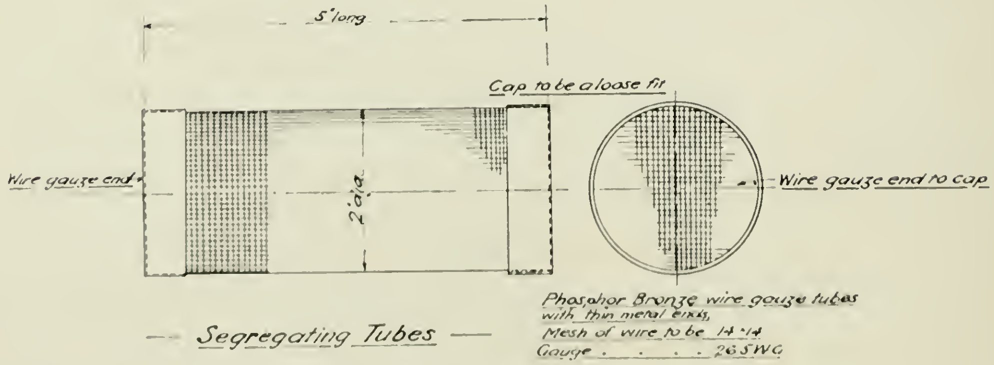


FIG. 25. PLAN OF SEGREGATION TUBE.

induce oviposition, but unless the rim of the jar fits quite accurately minute house ants, belonging to the group *Leptothorax*, may gain entrance and kill the mosquitoes. If an accurate record of the number of eggs deposited is desired, care must be taken to search the rim of the jar just above its point of contact with the blotting paper, as eggs are frequently deposited or stranded in this position.

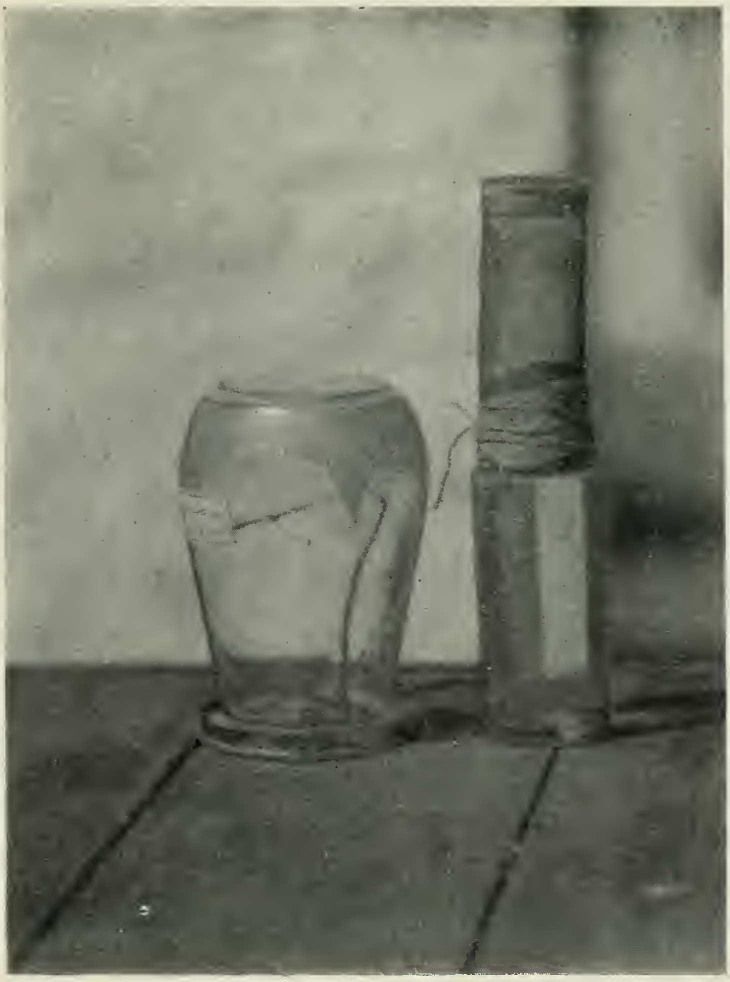


Plate No. 28.

Small Cage made from a hurricane lamp chimney, also method of arranging wire gauze tube over jar containing pupæ in order to avoid waste of time in transferring adults.

For *Stegomyia simpsoni* and *S. luteocephala* some larger and more elaborate arrangement of a small pool was generally needed before the females could be induced to lay, and the latter species seems to greatly prefer ovipositing on or in close proximity to a surface of wet wood.

When eggs are required in numbers it is best to keep a number of mosquitoes of both sexes in a large cage. A convenient method of collecting the eggs of *Stegomyia* under these circumstances is to place a pan of water in the cage with slips of filter or blotting paper arranged so that one end dips into the water and the other is turned back over the edge of the pan ; by adopting this method the eggs can easily be removed and counted without disturbing them. The water in the pans should be allowed to slowly evaporate, so that the eggs are left above water level, and do not hatch as would be the case if the pans were re-filled. If eggs laid on a wooden surface are required, the sides of a willow chip box answer excellently, as the wood is absorbent and of light colour, and its curvature is retained sufficiently to allow of its standing upright upon its edge in a shallow pan of water.

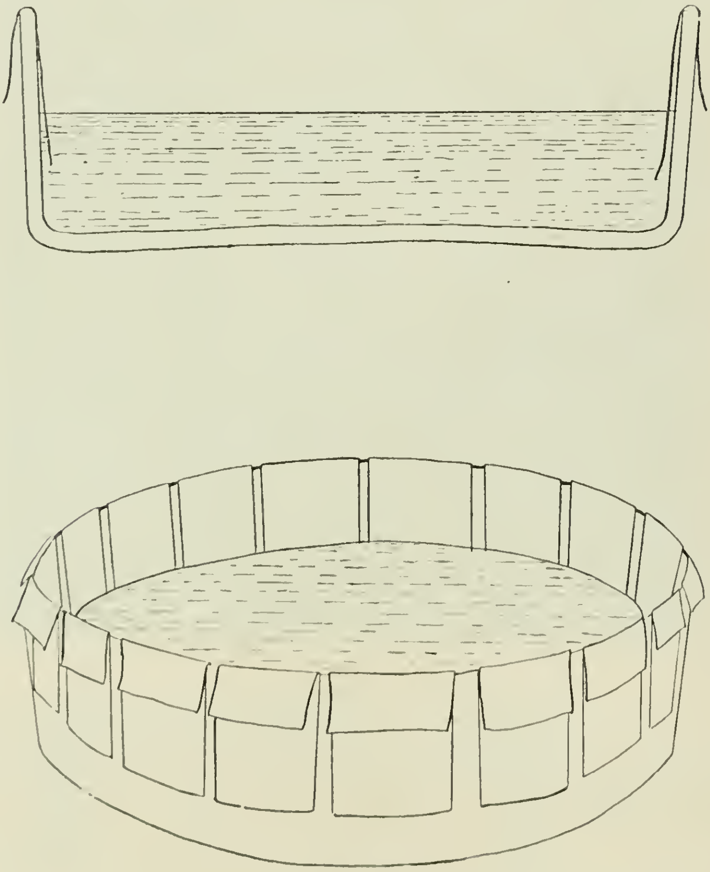


FIG. 26.

Hatching.—To test the hatching capacity of eggs laid by separate females or different batches of eggs laid by a single individual requires daily observations, which may have to be continued for weeks together, and necessarily consume a great deal of time. If it is desired to submit the eggs to identical conditions the different batches will have to be kept separated from each other in the *same* pan of water. Two methods of fulfilling these conditions were employed. In the first the eggs were carefully submerged in small glass sporulating dishes, which were in their turn placed at the bottom of a large pan of water. It was found necessary to check the number of larvæ captured in the pan each day against the number of empty egg shells, and to do this the sporulating dishes had to be taken out of the pan for examination under a low-power microscope and replaced.

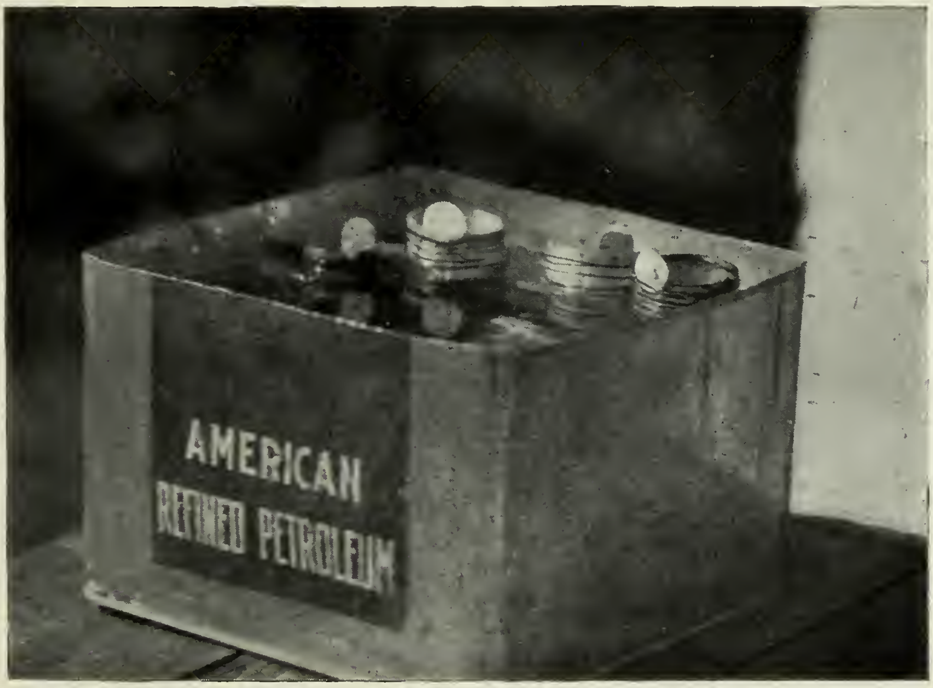


Plate No. 29.

Floating Docks. Method of testing the hatching of different batches of eggs under identical conditions.

Objections to this method were (1) that the eggs had to be completely submerged, and it has been stated, by Goeldi (see Carnegie Monograph, "Mosquitoes of North America," page 282), that continued submergence kills the eggs. Death rate from this cause is not by any means obvious in some of the experiments, but where *any* mortality at all has occurred, it is impossible to prove a negative. (2) Great care had to be taken in removing and returning the small dishes to the large pan, in order to avoid scattering the eggs and mixing them. (3) The gradual accumulation of dirt and organic growth tended to obscure a free view of the eggs after two or three weeks' immersion.

An alternate method was devised of keeping the eggs in small floating docks (*see* Plate No. 29, page 177) with a fine gauze bottom made as follows. The top is taken out of the lid of a willow chip box, and the hoop is used to stretch a piece of motor veil (silk if possible) over the top of the box. Thread is wound round to prevent the box from coming to pieces when the water melts the glue with which it is fastened together. The bottom of the box is removed by soaking it in water, and three cork floats are pinned on to the sides at the level at which the dock is required to float.

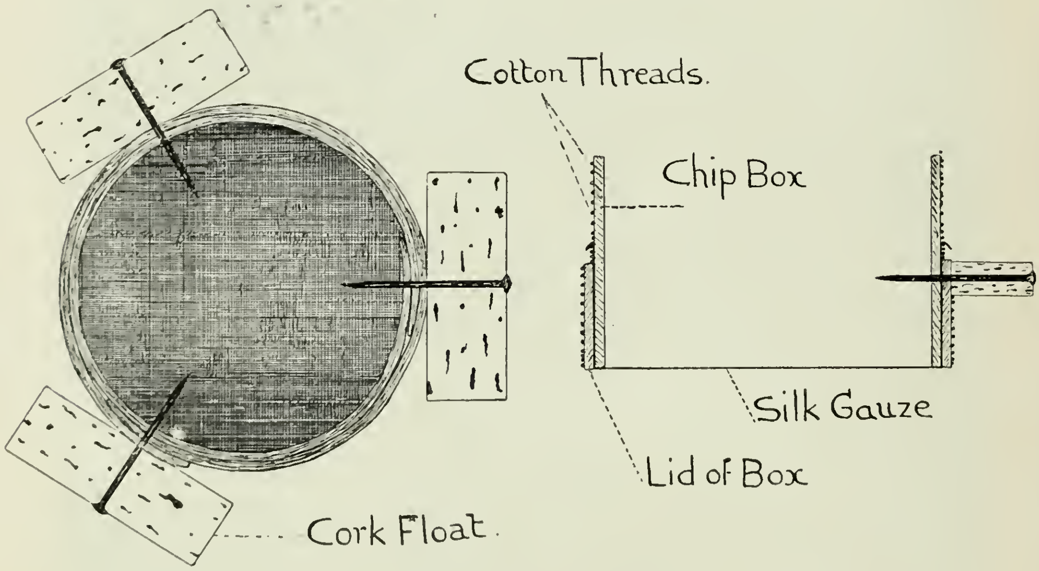


Fig. 27. Plan and Elevation of Floating Docks.

By adopting this plan examination is facilitated, the necessity of submerging the eggs is avoided, and a great saving of time is effected, as the sinking of floating eggs is a tedious process. Its defects are that with long continued use the gauze rots and the wood and cork become water-logged and lose their bouyancy. The saving of time is so great, however, that the plan is strongly to be recommended in all cases where it is not necessary to carry the hatching test to its ultimate conclusion of waiting until every single egg has had a complete chance of hatching. In most cases it is practical to close the experiment at a definite date and open the remaining eggs to see if they are dead or contain living larvæ.

Methods with larvæ.—For sorting out and transferring newly hatched larvæ a pipette with a rubber teat affords an obvious and speedy method. It may also be used for larger larvæ and pupæ, but care must be taken with the latter that the aperture is sufficiently large, otherwise they are easily injured.

When counting or catching newly hatched larvæ in a glass dish, the use of a black card or paper placed beneath it is helpful and, if the dish is supported above instead of resting *on* the black surface it renders observation still easier. With older larvæ, that have fed, a pale background is just as effective, owing to the dark colouration of the contents of the alimentary canal.

Larvæ and, to a less marked extent, pupæ of *Stegomyia* always avoid light; advantage may be taken of this when they are in a glass receptacle to make them collect in a corner or to one side for the purpose of rapid capture.

Breeding pans should not be placed in the sun or a strong light, otherwise they develop a growth of green algæ which appears to be detrimental, possibly by way of competition, to the development of the insects.*

If it is desired to breed *Stegomyia fasciata* free of gregarine parasites care must be taken to collect the eggs with a camelhair brush as soon after laying as possible and transfer them to clean filter paper. It is probable, if the sporocysts are voided by the females as suggested by Ross, that the young larvæ may ingest them at their first meal immediately after hatching.

Breeding pans in large cages containing a number of insects, especially when their numbers are recruited from several sources, are almost certain to become infected in a short time, and will remain permanently infected unless frequently cleaned.

For rearing the larvæ almost any sort of organic matter put into the breeding pans will serve, though care should be taken to thoroughly adjust the quantity to the size of the pan and numbers of the larvæ. Any sort of grain, dead insects, fæces of animals, fallen leaves, or other vegetable refuse, blood clots, etc., have all given good results.

Methods with adults.—To obtain a prompt supply of adults, dark coloured pupæ should be selected for preference, as the darkening signifies that the adult is nearly ready to emerge. To avoid waste of time in catching and boxing or caging mosquitoes for experiment, it pays to capture and segregate pupæ in jars of such size that an easy junction between the box or small cage and the rim of the jar with a slip of paper is possible (*see* Plate No. 28, page 175). One of the advantages obtained, apart from the saving of time, is that the age of the specimens is accurately known to within a few hours.

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* Experiments now in progress suggest another possibility, *i.e.*, that strong light or sunshine checks the growth of bacteria.

For infection experiments the clipping of one or both the wings is a reasonable precaution. To do this it is necessary to use chloroform ether or some other vapour to stupify the insects. Under cool conditions, temperature up to 65° F., ether causes a lower death rate than chloroform, but with a temperature of 80° F. chloroform answers best. After the operation it is well to place the insects in a warm incubator up to blood heat, or, failing this, in a strong draught, as this expedites their recovery and lowers the mortality resulting from the use of the vapour.

In dissecting infected mosquitoes which prove refractory to the usual methods of manipulation, a dissecting needle ground to a triangular section may be run up between the body wall and the internal organs from anus to head. Another needle is then drawn along one of the sharpened angles, thus slitting the insect longitudinally. With a little practice the gut and stomach of insects, which have recently gorged themselves with blood, may be extracted without rupture in this way. The method is also applicable and very useful when making dissections of any small grub like insect larvæ—one of its advantages being that the skin can be spread out flat and mounted independently so that its external surface with spines, setæ, tubercles and spiracular openings can be examined with care.

XIV.—SUMMARY.

MOSQUITOES AT FREETOWN.

I.—Introductory Notes.

Judged by sight or feeling mosquitoes are scarce in Freetown, and though a round with one of the Inspectors of the Sanitary Department may correct this view in so far as the larval stage is concerned, the increase in numbers of both larvæ and their breeding places *outside* the town area afforts eloquent testimony to the efficiency of the Sanitary Administration.

Within the central area the larvæ of *Stegomyia fasciata* take precedence, followed by those of *Culiciomyia nebulosa*, which species appears a spasmodic breeder in comparison with the persistency of *S. fasciata*. *Culex decens* is also not uncommon. *S. simpsoni* breeds in water in the axils of the leaves of plants growing within the town, as do also *Uranotœnia ornatus* and *Eretmopodites dracœnœ*. *S. luteocephala*, *Ochlerotatus apicoannulatus*, *O. minutus*, *O. leucarthrius*, *Eretmopodites quinquevittatus* and *Culex nigripes* are also occasional breeders within the town area. Of the species of *Anopheles*, a few larvæ of (*Pyretophorus*) *costalis* only were taken in the town, but in the surrounding district this species breeds freely, being frequently present in rock pools with swarms of *S. sugens.* The breeding places of (*Myzomyia*) *funestus* are extensive areas of shallow trickling swamps lying east and west of the town.

II.—Bionomics of Stegomyia fasciata.

(A.) EGGS.

(*a.*) *Shape and structure.*—The dark spindle-shaped eggs of *S. fasciata* vary considerably in shape and size, affording specimens which approximate closely in general appearance to those of the allied *S. sugens*, *S. luteocephala* and *S. simpsoni*, all of which differ appreciably among themselves.

The bosses with which the eggs are studded are not mere structural excrescences of the shell, but are pockets containing small masses of a substance which, unlike chitin, is readily stained by hæmatoxylin; these bosses are possibly connected with the ability which the eggs seem to possess of replacing moisture lost through desiccation.

(*b.*) *Laying.*—If actually laid on the water surface they are usually deposited so close to the margin as to become stranded by capillary action on the sides of the pools, or on partially submerged objects, such as dead leaves, lying in it. In many instances eggs were laid on wet surfaces quite beyond the limit of capillary action.

(*c.*) *Hatching and the conditions which control it. Experiments I. to XVIII.*—Incubation seems to invariably follow laying within 30 to 40 hours, at the Freetown temperature, but the hatching of any given batch may be distributed over a lengthy period. Apparently the eggs are endowed with a complex constitution, which allows of the whole of a batch hatching together, so soon as they are placed in water, or of a scattered emergence of the larvæ consistent with the view that some internal machinery which produces zonal or sectional hatching has been called into action. Experiment No. XVIII. suggests that the latency or activity of this machinery is determined by the conditions of humidity or drought which prevail *after* incubation. When a batch of dried eggs is placed in water a certain proportion generally hatch within a few minutes or hours, but others, apparently belonging to the same hatching zone, may resist the first or second immersion, yielding to some subsequent one. Similar powers of resistance will probably be shown by a proportion of the eggs in each hatching zone of a given batch.

After a series of experiments, Nos. IV. to XIII., in which various possible factors were tested, evidence was obtained that cooling to the extent of some 5° or 10° F. acted as a stimulus to induce the hatching of eggs that would otherwise have deferred doing so (Experiments Nos. XIV. to XVI.). A response to cooling accords well with the needs of the species, as a distributed emergence of larvæ so arranged allows of full advantage being taken of the increased facilities afforded for breeding in small and rapidly drying pools with the advent of rain.

Experiment No. XIX.—Agitation of the water, mentioned by Mitchell* as a factor in the hatching of eggs, failed to give any decisive result.

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* "Mosquito Life," page 26.

Bacterial action would appear also to be an important factor in hatching and in some instances to be essential (*see* footnote on page 51).

(*d.*) *Period of viability. Experiment No. XX.*—The period during which the eggs retain their viability would seem to be to some extent conditioned by temperature, humidity and ventilation, but, owing to the destruction of stored eggs by book-lice (*Psocidæ*) (Experiment No. XXII.), no adequate statistical evidence on this point was obtained. The longest period of viability was 262 days; in another test large numbers hatched after 200 days' dry storage. When kept continually immersed some eggs did not hatch for periods of from two to five months. (Experiments Nos. V., VI. and VII. show examples).

(*e.*) *Temperatures which the eggs are able to survive. Experiment No. XXI.*—Eggs brought back from Freetown and those laid in England hatched after exposure to 28° F. and 30° F. for 24 hours, but none hatched after an exposure of 25 days. 24 to 28 hours at 96° F. did not greatly, if at all, affect the percentage hatching at Freetown. In the experiment carried out at the Lister Institute there was a marked difference in the hatching percentage of Freetown eggs and those laid in London after exposure to 96° F. A few hatched after 24 hours' exposure to 102° F., but all the eggs of a batch placed at 108° F. for 24 hours failed.

(*f.*) *Enemies of eggs. Experiment No. XXII.*—The only active enemy discovered was a species of book-lice (*Psocidæ*) which wrought immense havoc among the stocks of stored eggs. Ants, considering the untiring activity of their search for the smallest particle of food, seemed strangely indifferent to the eggs.

(*B*) LARVÆ AND PUPÆ.

The larval period is conditioned by temperature and food, breeding tests dealing with the latter factor only were practicable at Freetown, although some trials concerning the survival limits in regard to temperature were carried out.

(*a.*) *Experiments Nos. XXIII. to XXV.*—A very wide range of organic matter will serve as food for the larvæ of *S. fasciata*, and the water may be so heavily charged as to become foul and thick, without harming the larvæ, so long as it remains free of scum, floating oil or fat.

(*b.*) *Period of larval life.*— Under the most favourable circumstances the larval life is passed within four days ; on the other hand, with a scarcity of food, it is prolonged for upwards of 70 days. In tap water of average purity the larvæ are unable to pass the first moult apart from added nutriment, but they continue to mark time until the death of their fellows enables a few to complete their development. Probably, in an uncovered breeding pool the fall of organic dust would be sufficient to afford food for a fair number of larvæ.

(*c.*) *Small adults reared from starved larvæ.*—Shortage of food results in the production of small sized adults—a somewhat important point on the practical side. Well-covered cleanly cisterns therefore require covers of specially small mesh wire gauze (not less than 18 × 18), there being always a danger that eggs will be washed in from gutters during rain.

(*d.*) *Interaction between larval growth and the development of bacteria. Experiments Nos. XXVII. and XXVIII.*—Experiments show an apparent association between the speed of larval growth and the development of bacteria. The scarcity of bacteria in the gut of larvæ taken from water swarming with these organisms is probably evidence of the speed with which bacteria are assimilated, as it seems improbable that, by the use of the mouth brushes, or any other means, the larvæ could avoid swallowing them. The marked clearing action of mosquito larvæ in foul water affords a parallel instance of interaction between them and the bacteria, although this might be a secondary effect due to the absorption of organic matter by the larvæ, and not the ingestion of the bacteria. Experiment No. XXVIII. affords evidence of this interaction, and shows that, when two similarly stocked beakers were prepared, in one of which bacterial action was given four days' start of the larvæ, the bacteria absorbed nutriment, which, even in the event of the larvæ devouring them, could not be regained.

(*e.*) *Temperature which the larvæ and pupæ are able to survive. Experiment No. XXX.*—Larval growth and pupal development proceeded normally in a large tin exposed to full sunlight—the temperature of the water rising to 103° on one occasion. Trials with larvæ and pupæ taken from this tin showed that the upper limit of temperature they could survive lay between 112° F. and 115° F. As regards cold, pupæ reared at 80° F. remained active at 50° F., but about half the larvæ submitted to the test became stiff and immobile at this temperature, quite irrespective of size. With a further reduction to 40° F. all the larvæ became immobile at the bottom, while the pupæ, with two exceptions, also lost all power of movement, but remained at the top. Both larvæ and pupæ recovered when the temperature was allowed to rise, but there was a mortality of 6 out of 40 larvæ and 2 out of 24 pupæ submitted to the test.

(*f.*) *Survival of larvæ and pupæ when submerged. Experiment No. XXIX.*—183 larvæ and 40 pupæ were submerged in a wire gauze tube of 18 × 18 mesh for 20 hours; 8 per cent. of the pupæ and 27 per cent. of the larvæ survived.

(*g.*) *Survival of larvæ on wet filter paper.*—Two larvæ in their fourth skins were stranded on a piece of filter paper which was kept continually wet; one lived 3 and the other 10 days.

(*h.*) *Enemies of the larvæ. Experiment No. XXXII.*—As pointed out by MacGregor,* the larger larvæ of *S. fasciata* apparently consume the smaller ones; mortality from this source seems to be limited to larvæ undergoing the first moult being ingested by those in their fourth instar. The habit of breeding in small collections of water, while saving the species from the attacks of many enemies, enhances the danger of fratricide, but the species is safeguarded from this risk to a considerable extent by the intermittent hatching of the eggs. Tadpoles, as stated by several authorities, certainly do not attack well-grown mosquito larvæ, though they probably have a checking influence, owing to competition for food. It seems possible, however, that the small larvæ undergoing their first moult, might be engulfed by tadpoles with other food. A small water-bug, found in rock pools, attacks the larvæ of *S. sugens*, and probably other species as well, but it was never seen in sufficient numbers to be a serious check. A species of *Ostracoda*, belonging to the genus *Cyprii*, seems to be inimical to mosquito larvæ, apparently as a competitor for food.

(*C.*) ADULTS.

(*a.*) *Pairing and feeding.*—In captivity there seems to be no regular precedence either of pairing or feeding; both functions are practised at any hour of the day or night—late afternoon being perhaps most favoured. The act of pairing commences during flight and occupies only a few moments, but is possibly repeated at frequent intervals. A single male is able to impregnate 10 females more or less effectually, and to fertilize 750 eggs (Experiments Nos. XLVI. and XLVII).

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* "Journal of Tropical Medicine and Hygiene," No. 17, Vol. XVIII, 1st Sept., 1915, page 195.

(*b.*) *Development of eggs. On a single meal of blood and also on blood other than human.*—In feeding satiety is evidently aimed at, and an interrupted meal is completed as speedily as possible ; a perspiring skin attracts more readily than a dry one. A single full meal of blood is sufficient for egg production in many cases, possibly for all, though the eggs are sometimes retained for many days. The blood of rats, dogs, goats and bandicoots seems just as effective for the development of the ovaries as human blood, but blood taken up from a living host seems to be a practical, though not a theoretical, necessity. *Fed on blood mixed with syrup. Experiments Nos. XLIV. and XLV.*—A single fertile egg was laid when the only food given was blood from a sheep mixed with syrup.

(*c.*) *Feeding in relation to egg laying.*—Females with ripe ovaries usually refuse to feed until they have deposited the bulk of their eggs, when they feed greedily. Females in their period of greatest vigour tend to develop and lay their eggs in masses at about three-day intervals, feeding on the first and second days after depositing their eggs, and fasting while the ovaries are full ; Experiment No. IX. and the Appendix to Experiment No. XLVI. are typical instances. The female used in the latter experiment laid 837 eggs in twelve batches, exclusive of odd eggs, while the former, in 22 days, laid 712 eggs in fifteen batches.

(*d.*) *Retention of eggs.*—Fertilized females that have only received a single meal of blood may retain their eggs for a considerable period before laying them ; Experiment Nos. XLI. and XLII. show examples.

(*e.*) *Choice of situation for depositing eggs.*—Tests showed that the widely-held opinion that the kitchen and boys' quarters are the most likely situations to look for *S. fasciata* was in the main correct, but it seems questionable if the preference is not as much due to the likelihood of there being foul water present, as to the chance of feeding on the boys. No eggs were ever deposited in a jar of clean water kept in the boys' bedroom.

In the mosquito house a wooden tub or tin pans were favoured as against a galvanized iron pail ; Experiment No. XXXIII.

(*f.*) *Baits to attract pregnant females.*—Experiment No. XXXIV. affords evidence which seems fairly conclusive of some selection by the females, but it was by no means final, and is as suggestive of subordination to their own tastes as to the needs of their progeny.

(*g.*) *Eggs not laid apart from wet surfaces. Experiment No. XLIII.*—Fertilized females regularly fed on human blood would not oviposit when no wet surface or water was available.

(*h.*) *Length of adult life. Experiment Nos. XXXV. to XL.*—The longest lived specimen was the female used in Experiment No. IX., which was killed by ants after 95 days. The longest lived male life was 50 days, in Experiment No. XXXVII. Average lives of 29 days for males and 44 days for females are shown in Experiment No. XXXVIII., the specimens being fed on syrup and kept in a still and, for Freetown, not very moist atmosphere. Specimens kept in wire gauze tubes give very much lower averages, and no evidence was obtained that *S. fasciata* can habitually, or is likely, save under very exceptional circumstances, to tide over the dry season in the adult stage.

(*i.*) *Enemies in the adult stage. Experiment No. XLVIII.*—Owing to its retiring habits, *S. fasciata* probably suffers more from wingless than winged foes once it has gained entrance to a dwelling. Ants proved themselves to be deadly foes to caged mosquitoes; two species of spiders, one a small web spinner, the other larger, spinning no regular web, practically lived on *S. fasciata* in the mosquito house, and a small flattened scorpion was also discovered, which ate large numbers when confined in the same box with it. Young mantidæ were also found to capture and devour them. Ants, spiders and, perhaps, the scorpion are probably serious enemies even to active, unconfined specimens; the continual waving of the raised hind legs of the resting insects is, I suspect, a measure of preparedness to avoid attack by wingless foes.

A slender wall-haunting lizard quickly cleared a large cage of *S. fasciata* and probably acts as a salutary check to their increase.

III.—Parasites.

The only parasites encountered were a gregarine *Lankesteria culicis* Wenyon and a species of yeast.

IV.—Sterility of the Pupal Gut.

Experiments carried out in collaboration with Dr. G. G. Butler, although not conclusive, point to the probability of the sterility of the pupal gut, in so far as infection by bacteria is concerned.

V.—Notes relating to the Life History of some Related and Associated Species.

S. sugens.—The eggs are similar in structure and hatching habits to those of *S. fasciata*—cooling acting as a stimulus; larvæ are quite as rapid in development; pupation may occur within three days of hatching. The adults have many traits which suggest that their habits are more primitive than those of *S. fasciata*, which shows the characteristic laxness which seems so frequently to follow the close association of animals with man.

S. simpsoni.—Captive females fed heavily on human blood, but showed a decided preference for feeding at night. No pairings were seen, nor were any fertile eggs laid. The breeding of specimens from a collection of dry leaves taken in a water-hole proves that the species is able to resist drought, and is undoubtedly fitted to pass the dry season in the egg stage.

S. luteocephala.—The remarks made in reference to *S. simpsoni* are generally applicable to this species. Only infertile eggs were obtained, but the insects showed a decided preference for ovipositing on wood. The larvæ show some ability to survive among wet leaves.

Ochlerotatus apicoannulatus.—This species is also fitted to survive drought in the egg stage; the females bite readily and occasionally fill themselves with human blood. Pairing was never witnessed, nor were any eggs laid.

O. minutus.—Females of this species bit frequently and occasionally fed well on human blood. No eggs were obtained.

Uranotænia ornatus.—Reared from larvæ could not be induced to feed at all, and survived for less than two days.

Eretmopodites quinquevittatus.—The females of this and other species of the genus occasionally feed greedily on human blood. Pairing took place in a very small cage. The eggs are laid in twos and threes and vary greatly in size, being usually much larger than those of *Stegomyia.* Some cement appeared to be used to attach them just at or below water level. Fertile eggs failed to survive drying, but others of the same batch, which were kept in water, duly hatched. The larvæ are of the *Stegomyia* pattern, but are even more slender in build, and in habit are confirmed bottom feeders. The fourth skin larva retains the habit of browsing on the sides and bottom of the breeding pan that is characteristic of young larvæ of *S. fasciata.*

VI.—Eggs laid on fallen leaves in water-holes.

Eggs are laid on fallen leaves lying in water-holes. The following species were bred from leaves taken from an empty water-hole in January and immersed in March :—

> 233 specimens of *S. fasciata* ; 24 of *S. simpsoni* ; 81 of *S. luteocephala*, and 14 *Ochlerotatus apicoannulatus*.

VII.—Meteorological conditions as affecting breeding and distribution.

The early tornadoes which herald the breaking of the dry season begin as dry squalls with but little rain, and would afford an efficient means of bringing mosquitoes from the outlying districts into Freetown ; this would apply especially to the *Anopheles* mosquitoes. At the onset of the rains breeding is necessarily restricted to a few pools in favourable situations ; later these will be rendered untenable, but the insects reared in them will distribute and find other smaller and more numerous pools available as the showers increase in number and intensity. The small number of places available for early breeding should make it possible to schedule and treat them at short and regular intervals at the very commencement of the rains, even if outside the bounds of the regular sanitary district.

VIII.—Experiments dealing with the destruction of S. fasciata.

Experiments Nos. XLIX., LI., LII., LIII.

Naphthalene at 1 in 4,000, under the conditions of Experiment No. LI. is an effective larvicide ; at 1 in 8,000 it is more effective than petroleum, but allows of a few survivors. Soft soap, at less strength than 1 in 1,000, is not an effective larvicide.

Emulsions of soft soap and petroleum are far more effective larvicides than either of them used separately, killing all larvæ and pupæ at 1 in 16,000 (water temperature about 80°, experimental pans covered with cheese cloth). With naphthalene added to the emulsion it was equally effective at 1 in 20,000.

As against submerged eggs petroleum and soft soap emulsion at 1 in 8,000 is not effective in killing the larvæ within the eggs. It has the result, however, of inducing a high percentage of the less resistant eggs to hatch at once, when the young larvæ are killed.

IX.—*Effect of salt water on larvæ, pupæ and eggs of S. fasciata.*

Experiments Nos. LIV. to LVII.

Salt water (from Freetown Harbour) speedily kills the larvæ of *S. fasciata* but does not destroy the pupæ.

As against the eggs, though it does not destroy them, it causes a high percentage of the less resistant ones to hatch at once and a considerable percentage of the specially resistant. The young larvæ, after hatching, are speedily killed by it. The use of salt water for flushing culverts and gutters and watering roads might, if practicable, prove very beneficial. The effect of salt water as a stimulus to hatching appears to be unconnected with its specific gravity ; a solution of sugar of the same specific gravity gave different results and both differed from the tap water control. Both solutions, however, were apparently responsible for a mortality of 7 per cent.

X.—*Size of the Mesh of Netting for use against S. fasciata.*

Adults can get through 10 × 13 cotton netting ; they also pass 11 × 15 of the same material, and 14 × 14 wire gauze. No evidence of normal specimens escaping through 16 × 16 wire gauze occurred, but there is little doubt that the dwarf specimens caused by scarcity of food could do so. As suggested by the authorities of the Panama Canal Zone, for safety a mesh of not less than 18 × 18 should be used.

XI.—*Methods and Apparatus.*

This section deals with the apparatus used in this research, and the methods adopted in carrying out breeding and other experiments.

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N° 5

A CONTRIBUTION TO THE BIONOMICS OF *PEDICULUS HUMANUS* (*VESTIMENTI*) AND *PEDICULUS CAPITIS.*

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In its inception this work was planned with a view to supplement our present knowledge of the life history of *Pediculus humanus* (*vestimenti,* which we owe so largely to Warburton[1] (1909). The scheme was to work out in detail certain problems relating to sex and fertility and incidentally to obtain further evidence in support of what is already known concerning the laying and hatching of eggs. The success of an attempt to breed *P. capitis* under conditions that had already proved satisfactory with *P. humanus* suggested that it was worth while enlarging the scope of the scheme in order to include the head louse, as this insect seemed to be amenable to the same conditions of captivity.

These conditions, as nearly natural for *P. humanus* as were consistent with captivity and isolation, are admittedly more artificial when applied to *P. capitis*; it is necessary to keep this fact in mind when comparing the bionomics of the two insects as described in this paper.

The lower egg production of *capitis* is in all probability chiefly due to the smaller egg-containing capacity of its body; for, in spite of the fact that the eggs of the head louse are slightly smaller than those of the species associated with clothing, the body extension of the ♀♀ *P. humanus* still gives them a marked advantage with respect to the number of fully developed eggs that they can carry. The shorter life, and apparently lower vitality of *P. capitis* as compared with *P.*

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[1] I was unaware of the publication of Sikora's (1915) excellent paper, which contains much fuller details than any hitherto published account of the biology of *P. humanus* (*vestimenti*), until the experiments detailed in this paper had been mapped out and half completed.

humanus, is probably due, at all events in part, to the method of feeding and other conditions of captivity.

Origin of the strains experimented with.

Lice were obtained from three separate sources; a London Borough Infirmary, one of the London County Council cleansing stations, and a Salvation Army shelter. I take this opportunity of recording my thanks to the Officials of these Institutions who so kindly assisted me by providing material.

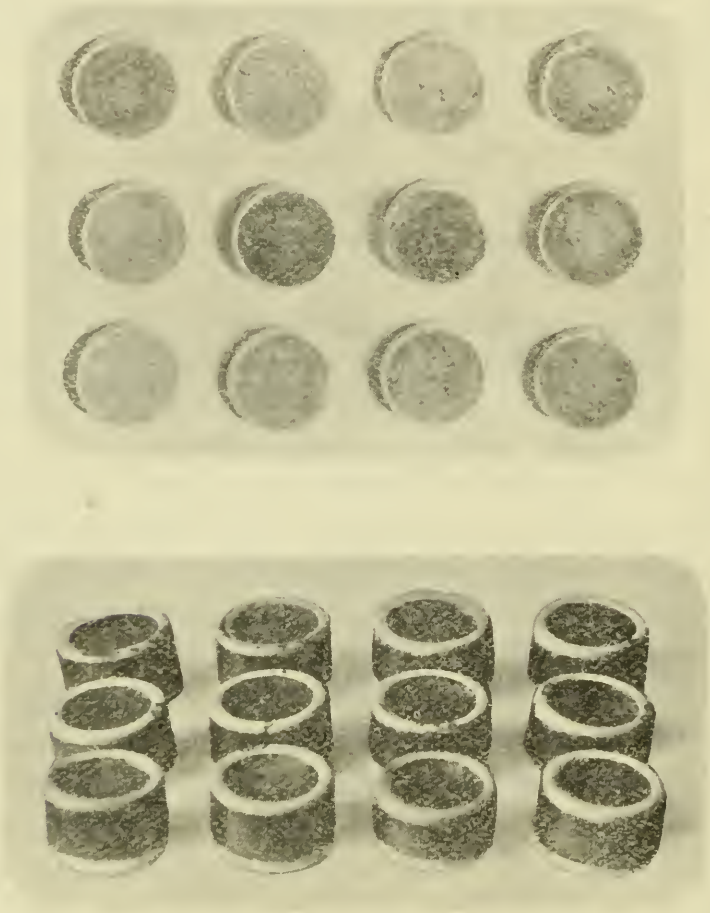
The insects obtained from these sources were placed together and treated as a single stock in the case of *P. humanus*. With *P. capitis*, however, two stocks were kept; one raised from nits on the hair and the other from active insects received with the hair. This course was pursued in order to avoid possible error due to a mixed infection, but as no difference in size, structure, or habits was observed between the two stocks, it was concluded, after several generations had been bred, that only *P. capitis* had been present. Subsequently either stock was drawn upon to supply insects for experiment.

Methods. The method of breeding employed was essentially the same as that adopted by the author when experimenting with fleas (Bacot 1914), in one particular adaptation to the circumstances it closely paralleled a feature of the method mentioned by Sikora (1915), the idea, however, was differently applied.

The insects were kept in glass-bottomed entomological boxes lined with a loose slip of cloth to give foothold. In the case of *P. capitis* a small tuft of hair was also generally added, although it was found not to be essential. The open top of the box was covered with chiffon, the lid being partly closed over it to keep it stretched while it was securely tied in position with fine thread; as an extra precaution against escape the box was nested in one of a larger size. Feeding took place readily through the chiffon when this was placed against a suitable skin surface. As it was necessary during the progress of the research to feed isolated individuals or families in as many as three dozen separate boxes at once, small ones of three quarters of an inch diameter were used. Holes punched in pieces of card into which the gauze covered boxes were inserted, enabled them to be kept in position against the body during sleep, with the aid of a flannel belt. The insects had the option of feeding at any time covering a period of six or seven hours during which the chiffon covered openings of the boxes were against the skin.

During the day the boxes were carried in the pockets of a waistcoat, the eggs laid being hatched in the same or similarly fitted boxes carried beneath the clothing.

The only section of the experiments in which this procedure was modified was in that dealing with the eggs of ♀♀ submitted to differential feeding (Table VIII) and the trials conducted to test variability in hatching (Table IX), in which it was thought desirable to keep the eggs at a constant temperature. In both these instances the eggs were laid on cloth and placed in glass tubes plugged with cotton-wool, the tubes being kept in a humid incubator having a constant temperature of 31° C.



Figs. 1 and 2.

Breeding method when a large number of segregated individuals or broods have to be fed at the same time.

Habits of the insects noted when reared under the conditions mentioned above.

P. capitis is much the more active insect of the two. In egg laying either species can adapt itself to cloth or hair. *P. capitis* will sometimes lay eggs on cloth, although there are hairs in the box. *P. humanus,* on the other hand, seldom if ever lays on hair while there is cloth in the box and when compelled to do so the females of this species appear to be less skilful in cementing, or careful in getting the long axis of the egg into alignment with the hair. When forced to lay on hair they not infrequently attach the egg at an angle to the axis of the hair.

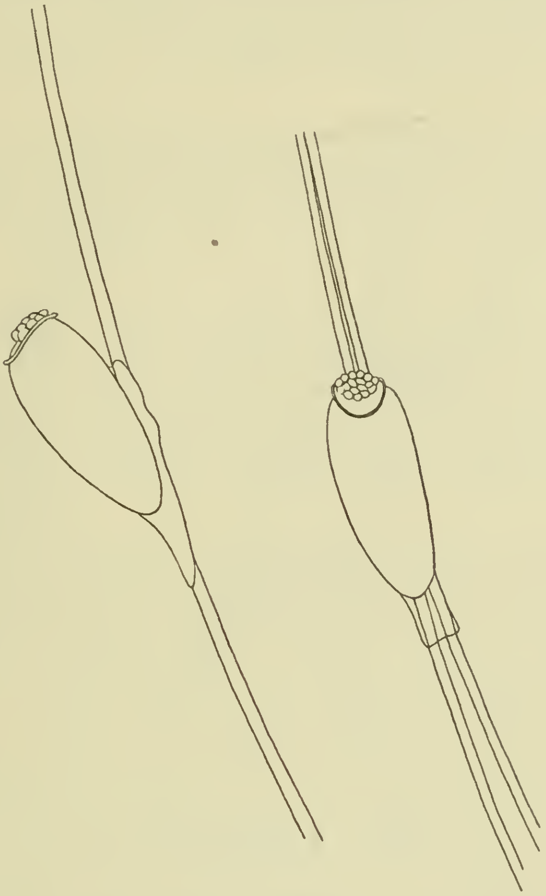


Fig. 3. *P. capitis* × 25.

Two or three ♀♀ and ♂♂ of *P. humanus* were placed in a small box with human hairs of two or three centimetres long from the forearm. The insects were liberally fed for three days, during this period they laid 35 eggs. Of these one was attached to the side of the box and was also cemented to the hairs which clung together owing to eggs having been fastened to two or more hairs at the angles of crossing. Nine eggs had been laid on the gauze cover of the box; only four eggs had been attached to the single hairs, while 21 (60 %) of the eggs were cemented to two or more of the hairs, the ♀♀ having apparently searched for positions where the hairs crossed or ran parallel to each other so as to avoid attachment to single hairs.

Several ♀♀ and ♂♂ of *P. capitis* were treated similarly, in this box—36 eggs were laid, all of them on hairs; 28 were attached to a single hair only; six had attachment to two and two to three hairs. A marked difference was observable, also, in the selection of position for attachment in the case of the eggs having attachment to more than one hair. In every case the hairs chosen not only ran parallel for more than the length of the egg, but in most cases they were side by side. The parallel hairs affected by *P. humanus* were separated from each other by a well marked space, while in most cases hairs crossing each other at a wide angle had been chosen.

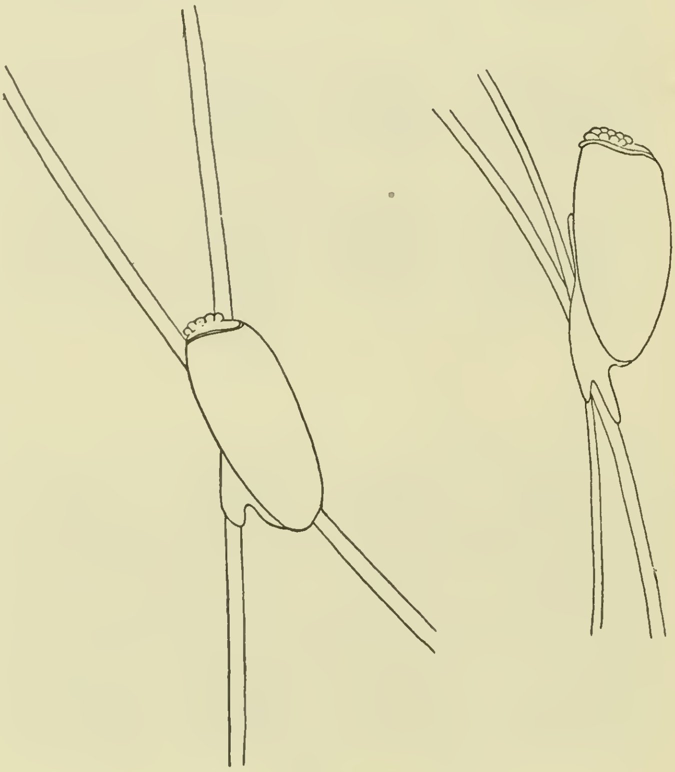


Fig. 4. *P. humanus* × 25.

P. humanus ♀♀ in most cases exhibited what may be roughly called a homing instinct; that is to say they returned again and again to the same spot to lay their eggs. As this habit made counting difficult, various methods of shifting the piece of cloth, turning it, so that the exposed surface went against the side of the box, the addition of a second piece of cloth, etc., were tried in an endeavour to get the eggs spread, instead of clustered. As a rule these attempts were unsuccessful, there seemed to be some attraction which led to the deposit of fresh eggs where others were already laid.

P. capitis ♀♀ occasionally exhibited the same tendency, but it was never so marked or "wilful" as with those of *P. humanus*. Another habit that was marked in the body louse and less noticeable but still to be observed with *P. capitis,* was a tendency to gregariousness shown by clustering, especially on the part of the larvae and nymphs when about to moult.

Pairing may be seen at any period of the day or night on the part of both species, the insects remaining together for a considerable time; periods of over an hour were observed, but I failed to ascertain what the limits were.

Both species show a very definite negative heliotropism, moving towards any shadow or dark coloured object in their vicinity. General observation suggested that their actions differed somewhat, however, according to whether they were at the time of exposure to light resting on a dark or a light surface. The following experiment was carried out with *P. humanus*:

Two eight inch squares of paper, one black and the other white, each having a sufficiently roughened surface to afford good foothold, were placed near together about two feet from a window. The lice at each trial being emptied out of a tube on to the centre of the paper.

Two batches of lice were used, each comprised five females and four males; eight trials were made on each paper—the batches being transposed from black to white and vice versa after each trial.

While these experiments strengthen the idea suggested by my general observations, viz. that there is a difference between the behaviour of the insects dependent upon their being on a light or dark surface when exposed to the light, their movements being less assured in the former case, the result is not so striking as I was led to expect. Had the papers been broader all the active insects would have reached the end farthest from the source of light, as all their tracks towards the sides were diagonals in this direction.

The sluggishness recorded is, with one exception, due to the inactivity of three ♂♂; of the 15 records 14 were due to the inaction of these three individuals.

No. of observation	Paper (black or white)	Distribution of lice on the paper		No. of observation	Paper (black or white)	Distribution of lice on the paper	
1	Black	3	*a*	9	Black	1	*a*
		0	*b*			1	*b*
		6	*c*			7	*c*
2	White	0	*a*	10	White	0	*a*
		3	*b*			3	*b*
		6	*c*			6	*c*
3	Black	0	*a*	11	Black	0	*a*
		1	*b*			0	*b*
		8	*c*			9	*c*
4	White	0	*a*	12	White	2	*a*
		3	*b*			0	*b*
		6	*c*			7	*c*
5	Black	2	*a*	13	Black	3	*a*
		2	*b*			0	*b*
		5	*c*			6	*c*
6	White	1	*a*	14	White	0	*a*
		3	*b*			3	*b*
		5	*c*			6	*c*
7	Black	0	*a*	15	Black	0	*a*
		1	*b*			1	*b*
		8	*c*			8	*c*
8	White	1	*a*	16	White	2	*a*
		2	*b*			2	*b*
		6	*c*			5	*c*

	Totals	
Black	9 = 13 %	*a*
	6 = 8 %	*b*
	57 = 79 %	*c*
White	6 = 9 %	*a*
	19 = 26 %	*b*
	47 = 65 %	*c*

NOTE. *a* = lice sluggish and failed to crawl.
b = lice crawled to side of the paper.
c = lice crawled to edge of paper farthest from the source of light.

Length of life of unfed lice under different conditions of temperature.

Lice at all stages of growth were taken from a stock box carried in a vest pocket and submitted to the following conditions: in a room, the air of which is very dry owing to central heating, temperature 16°–18° C.; in a humid incubator at 24·5° C. constant; in a dry air incubator at 37° C. constant.

At 16°–18° C. most of the insects died within four days; two lived five days; one adult was still living on the seventh day.

At 24·5° C. all died within five days.

At 36·1° C. all died within three days.

Newly hatched lice that had not been fed lived less than 24 hours at 36·1° C. When kept unfed in a box in the vest pocket newly hatched lice lived but little more than a day; none survived a second day.

Adults kept in a box unfed in the side pocket of a coat lived five days.

Cold. Active lice in all stages were placed in a cloth-lined box and kept in a cold room at −2·3° to −1·1° C. for 48 hours; all were stiff and motionless when taken out, but after 24 hours in a vest pocket all revived; they were afforded ample opportunity of feeding, after which they were again kept in the cold room for seven days; all were dead on removal.

Feeding. Hungry lice do not as a rule wander, but settle down to feed at once if placed on a suitable skin area. When a number are placed on a small area of not more than a square inch, there is a considerable difference in the time taken to draw blood; many obtain it within half a minute to a minute, others within two or three minutes, while a few may be five minutes or over. Such delay seldom if ever occurs with bugs (*Cimex lectularius*) presumably owing to their more powerful pumping apparatus, or possibly the greater depth of the wound. Fleas not infrequently fail at the first attempt, in which case they usually shift and try again at another spot. Lice, however, rarely if ever shift, but wait patiently until, presumably, the irritation caused by the injection of saliva dilates the capillaries and brings the flow of blood to the wound. This reliance upon the salivary fluid fits in well with the fact of the more intense and lasting effect of the bite, as compared with that of either fleas or bugs (on the author). With insects having such a generally restricted range of operations there is a very fair chance of their feeding a second time on the same area and benefiting by the inflammation resulting from their previous attacks.

Experiment shows that there is much the same variation in the time required to obtain blood on the part of newly hatched larvae as with larvae in their second skin, nymphs and adults; and the speed with which they fill their crops also varies from about two to fifteen minutes.

Growth and moulting: Pediculus humanus (vestimenti).

Forty newly hatched lice were kept in a box in a vest pocket and afforded opportunity for feeding during six or seven hours each night; cast skins were found as under:

	Number of days after hatching													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Number of cast skins			1	17	22		6	29	5	2	1	22	13	2
				1st moult				2nd moult				3rd moult		
			3 %	42 %	55 %		15 %	72 %	13 %	5 %	3 %	55 %	32 %	5 %

TABLE I. *Fertility of* Pediculus humanus (vestiment

Insects in the larval stage were taken from a stock box and reared until maturity in separate boxes. The ♀♀ wer
(approximately 7 hours). The ♂ used for the first pairings matured on 23 Dec. 1915 and died on 24 January 191
kept in separate boxes. None of them hatched.

The boxes containing the ♀♀ and eggs were carried in a waistcoat pocket during the day; at night they were in still

Reference No. of ♀	Date when ♀ matured	Date when the ♂ was added	Copulation observed	Date of removal of ♂	Days counting from maturity of the ♀ to that on whi														
					1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15t
1	23. XII. 15	23. XII. 15	—	24. XII. 15	—	—	—	—	8	10	4	6	5	6	7	7	4	10	4
2	23. XII. 15	24. XII. 15	—	25. XII. 15	—	—	—	—	5	7	4	7	3	7	8	8	3	7	7
3	23. XII. 15	25. XII. 15	26. XII. 15	26. XII. 15	—	—	*2*	—	6	4	5	5	7	4	10	6	7	3	7
4	23. XII. 15	26. XII. 15	—	27. XII. 15	—	—	*1*	—	—	11	5	7	6	3	11	8	5	7	
5	23. XII. 15	27. XII. 15	—	28. XII. 15	—	—	*2*	—	*4*	1	—	—	—	—	—	—	—	—	—
6	24. XII. 15	28. XII. 15	—	29. XII. 15	—	—	—	*2*	*3*	5	6	5	6	7	10	3	6	7	
7	24. XII. 15	29. XII. 15	29. XII. 15	30. XII. 15	—	—	—	*3*	*3*	*4*	4	5	6	6	5	8	5	6	
8	25. XII. 15	30. XII. 15	—	31. XII. 15	—	—	—	*2*	*3*	*2*	3	4	3	6	2	6	3	2	
9	26. XII. 15	31. XII. 15	1. I. 16	1. I. 16	—	—	—	—	*2*	*3*	3	6	4	5	7	8	6	2	
10	26. XII. 15	1. I. 16	—	2. I. 16	—	—	*2*	*3*	*5*	*1*	*4*	6	1	5	5	3	6	5	
11	28. XII. 15	2. I. 16	—	3. I. 16	—	—	*1*	*3*	*4*	*6*	5	6	6	5	9	9	2	6	
12	28. XII. 15	3. I. 16	—	4. I. 16	—	—	—	*1*	*2*	*4*	*4*	3	4	3	7	4	5	3	
13	30. XII. 15	4. I. 16	—	5. I. 16	—	—	*1*	*4*	*5*	*3*	7	8	5	8	4	6	10	7	
14	1. I. 16	5. I. 16	5. I. 16	6. I. 16	—	*1*	*3*	*4*	*4*	5	6	8	5	6	8	7	6	7	1
15	6. I. 16	6. I. 16	6. I. 16	10. I. 16	—	—	—	5	3	4	7	2	10	3	8	4	7	8	
16	10. I. 16	10. I. 16	10. I. 16	13. I. 16	—	—	—	3	3	7	8	5	6	6	10	4	6	9	
17	13. I. 16	13. I. 16	—	15. I. 16	—	—	3	5	4	5	7	7	4	10	4	7	8	8	
18	15. I. 16	15. I. 16	—	19. I. 16	—	—	—	5	7	6	7	8	4	8	6	10	8	6	
19	19. I. 16	19. I. 16	19. I. 16	20. I. 16	—	—	—	3	5	5	7	7	4	8	7	6	10	2	1
20	20. I. 16	20. I. 16	—	22. I. 16	—	—	3	5	5	7	6	8	5	3	8	3	9	8	
21	22. I. 16	22. I. 16	—	23. I. 16	—	—	1	4	5	6	7	6	7	7	6	10	—	9	

NOTE. The figures in heavy type indicate that these eggs were kept in separate boxes from those laid ea
of laying before this test was made was progressively shortened in order that the oppo

rticulars of pairing and egg laying.

fed in these separate boxes, the ♂ being placed with each ♀ in rotation. Opportunity for feeding was given each night
lays). Eggs laid before the introduction of the ♂ are italicised and were, in the case of ♀♀ Nos. 6, 7, 10, 12, 13 and 14,

tion to the human body.

laid. The number of eggs is indicated by the figure below																		Total eggs laid	
18th	19th	20th	21st	22nd	23rd	24th	25th	26th	27th	28th	29th	30th	31st	32nd	33rd	34th	35th	per ♀	Remarks
2	*9*	*2*	7	4	5	—	—	—	—	—	—	—	—	—	—	—	—	118	Died on the 23rd day.
4	*9*	*5*	*8*	*5*	*6*	*7*	2	5	4	3	5	1	—	1	4	4	—	150	Died on the 34th day.
8	*7*	*5*	*5*	*4*	5	5	7	3	6	4	4	5	3	6	4	6	2	166	See further entries after second pairing, Table II.
3	*6*	*6*	*8*	7	5	7	9	5	9	7	5	4	3	3	—	—	—	172	Died on the 32nd day.
—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	7	Died on the 11th day. Eggs infertile.
10	*5*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	95	Lost on the 19th day.
6	*6*	*3*	*4*	*5*	*2*	*9*	—	4	4	3	6	—	1	—	—	—	—	123	Died on the 32nd day.
3	*2*	*6*	*2*	*5*	*3*	*1*	—	—	—	—	—	—	—	—	—	—	—	70	Accidentally killed on the 23rd day. Eggs infertile.
7	*5*	*12*	*3*	*2*	*7*	6	7	6	6	5	5	5	4	7	3	—	—	159	See further entries after second pairing, Table II.
6	*6*	*5*	*3*	*8*	*5*	*2*	*1*	*6*	*4*	1	—	—	—	—	—	—	—	102	Died on the 29th day.
9	*7*	*11*	*14*	*7*	*9*	8	6	5	5	7	3	10	—	—	—	—	—	180	Died on the 30th day.
3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	55	Died on the 19th day.
7	*8*	*3*	*4*	*4*	*5*	6	4	5	4	6	5	5	4	—	—	—	—	154	See further entries after second pairing, Table II.
7	*6*	*6*	*6*	*2*	*3*	*10*	9	6	6	10	6	4	3	7	—	—	—	181	,, ,, ,,
4	*9*	*3*	7	5	7	8	5	5	4	10	—	—	—	—	—	—	—	148	,, ,, ,,
9	8	5	5	6	7	6	7	—	—	—	—	—	—	—	—	—	—	137	,, ,, ,,
7	*3*	6	7	8	6	7	7	—	—	—	—	—	—	—	—	—	—	141	,, ,, ,,
5	9	8	8	9	9	7	—	—	—	—	—	—	—	—	—	—	—	152	,, ,, ,,
5	8	5	6	—	—	—	—	—	—	—	—	—	—	—	—	—	—	115	,, ,, ,,
7	6	8	5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	117	,, ,, ,,
1	*10*	*5*	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	110	None of these eggs hatched, presumably this ♀ was not fertilized as she laid fertile eggs after pairing with another ♂, see entries on Table II.

r to test if the ♀♀ still retained the power of fertilizing them. It will be noted that with experience the period
, second pairing might take place before the ♀ was too old and feeble to profit by it.

Table I gives the record of 21 ♀♀ of *P. humanus* which were segregated in the larval stage and on reaching maturity were placed in rotation with a single ♂. Of these 21 ♀♀ 18 were more or less effectively fertilized by the one ♂. It will be noted that the ♀♀ invariably commenced oviposition irrespective of their having paired; probably egg development is entirely a question of nutrition and laying is completely automatic while feeding continues at a favourable temperature. In no case did eggs laid by virgin ♀♀ hatch.

As regards the three ♀♀ which laid only infertile eggs—it is probable that the death of No. 5 was connected with pairing, as she laid but one egg after the introduction of the ♂, and she died with the whole body and limbs as far as claws tinged with red. I suspect that death resulted from rupture of the alimentary canal due to violence during the sexual act. Deaths showing a similar post-mortem appearance are by no means uncommon. In the course of the experiments it was remarked that death frequently followed, if it did not actually occur during, the act of pairing if the ♀♀ were nearing their age limit.

In the case of No. 8 it is doubtful if pairing ever took place; this ♀ proved a poor egg layer, but the evidence available from the other ♀♀ which laid prior to pairing precludes any suggestion that low egg production resulted from infertility; it is possible, however, that both the failure to pair and small egg laying capacity were the outcome of a low vitality.

Probably the ♂ was too feeble to pair with No. 21, as he died the following day. Table V shows that there was no question in regard to the vitality of this ♀ as she laid fertile eggs after she had been placed with another ♂.

It was obvious early in the course of the experiment that ability of the ♀♀ to lay fertilized eggs after the removal of the ♂ did not continue for life (see Table IV). In order to test the period, the ♀♀ were removed to fresh boxes at a progressively shortened interval of time. The eggs laid in the second box are indicated by printing the figures in heavy type. By this means a general indication of the duration of fertility was attained; in the cases where eggs hatched which had been laid in the second box, showing definitely that fertility was still retained, the term "at least" indicates that the number of days might have been more. In several cases the number of days has been calculated by counting the number of eggs laid in sequence up to the number which hatched and reckoning the number of days from the removal of the ♂ up to the date of the last laid egg included in the total. In both cases it is probable that

TABLE II.

Pediculus humanus (vestimenti). *Particulars of egg laying after a second ♂ had been placed with the ♀♀.*

A ♂ that matured on the 1st January, 1916 was placed with ♀ No. 3 on the 26th January, he was afterwards placed with ♀♀ Nos. 9, 13, 14, 15 and 16, he was lost on the 5th February 1916. A ♂ taken from the stock box, of uncertain age, was placed with the later ♀♀ Nos. 17—21.

Reference number of ♀	Day, counting from maturity of ♀, when the second ♂ was introduced	Date on which the second ♂ was introduced	Day of copulation when observed	Day when ♂ was removed	Day, counting from maturity of ♀, on which eggs were laid after the introduction of the second ♂																									Total eggs laid per ♀	Remarks
					20th	21st	22nd	23rd	24th	25th	26th	27th	28th	29th	30th	31st	32nd	33rd	34th	35th	36th	37th	38th	39th	40th	41st	42nd	43rd	44th		
3	35th	26. I. 16	35th	36th	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	6	4	3	—	—	—	—	—	—	13	Died 38th day.
9	33rd	27. I. 16	35th	35th	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4	—	—	—	—	—	—	—	—	—	—	4	,, 35th ,, whilst pai
13	31st	29. I. 16	—	34th	—	—	—	—	—	—	—	—	—	—	—	—	3	2	—	—	—	—	—	—	—	—	—	—	—	5	,, 33rd ,, eggs infer
14	32nd	1. II. 16	—	33rd	—	—	—	—	—	—	—	—	—	—	—	—	—	5	7	6	7	5	7	5	4	10	—	—	—	56	,, 41st ,, ,,
15	28th	2. II. 16	—	29th	—	—	—	—	—	—	—	—	—	6	6	5	4	3	5	4	4	1	—	—	—	—	—	—	—	38	,, 37th ,, ,,
16	25th	3. II. 16	—	27th	—	—	—	—	—	—	6	10	6	4	9	6	6	4	10	7	8	4	—	—	—	—	—	—	—	80	Placed with a third ♂ on day and fertile eggs see Table III.
17	25th	6. II. 16	—	26th	—	—	—	—	—	—	7	9	3	10	8	7	4	8	4	7	6	2	7	3	—	10	3	5	5	108	Died 44th day.
18	24th	7. II. 16	—	25th	—	—	—	—	—	8	9	8	9	6	8	8	5	7	8	9	7	4	8	—	8	11	7	—	—	130	Placed with a third ♂ on day, see Table III.
19	21st	8. II. 16	21st	22nd	—	—	7	7	8	6	6	7	5	7	3	2	—	—	—	—	—	—	—	—	—	—	—	—	—	58	Died 31st day.
20	21st	9. II. 16	—	22nd	—	—	7	6	8	5	6	4	10	7	8	7	9	7	6	3	3	6	4	2	—	—	—	—	—	108	,, 39th ,,
21	19th	10. II. 16	20th 23rd	24th	—	7	7	3	11	4	6	11	5	5	7	10	10	8	—	—	—	—	—	—	—	—	—	—	—	94	,, 33rd ,,

TABLE III.

Pediculus humanus (vestimenti). *Particulars of egg laying after a third ♂ had been placed with the ♀♀.*

♂♂ taken from stock were used, age uncertain.

Reference number of ♀	Day, counting from maturity of ♀, when the third ♂ was introduced	Date on which the third ♂ was introduced	Day of copulation when observed	Day when ♂ was removed	Day, counting from maturity of the ♀, on which eggs were laid after the introduction of the third ♂								Total eggs laid per ♀	Remarks
					38th	39th	40th	41st	42nd	43rd	44th	45th		
16	37th	15. II. 16	—	not removed	3	1	—	—	—	—	—	—	4	Died 39th day.
18	42nd	25. II. 16	—	up to time of females death	—	—	—	—	—	2	8	3	13	Died 46th day, eggs infertile.

TABLE IV. *Fertility of* Pediculus humanus (vestime

Note. There is no necessary relation between the lice emerging on any particular day and the eggs la other; they were kept in the box with the laying ♀ until she was transferred to a new box. The

Reference number of ♀	Date when ♀ matured	Date when the ♂ was added	Days counting from date on which the																	
			7th	8th	9th	10th	11th	12th	13th	14th	15th	16th	17th	18th	19th	20th	21st	22nd	23rd	24th	5th
1	23. xii. 15	23. xii. 15	—	—	—	—	1	1	3	3	3	7	4	5	6	9	8	6	4	—	—
2	23. xii. 15	24. xii. 15	—	—	—	—	—	1	3	4	6	3	7	6	7	5	6	4	5	—	1
3	23. xii. 15	25. xii. 15	—	—	—	—	—	3	2	7	5	6	6	7	5	7	5	5	4	10	7
4	23. xii. 15	26. xii. 15	—	—	—	—	—	3	8	5	6	2	6	6	11	5	3	1	—	—	—
5	23. xii. 15	27. xii. 15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
6	24. xii. 15	28. xii. 15	—	—	—	—	—	2	4	6	4	8	12	7	8	3	2	—	1	—	—
7	24. xii. 15	29. xii. 15	—	—	—	—	1	3	3	5	4	6	5	8	2	4	9	6	8	—	1
8	25. xii. 15	30. xii. 15	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	26. xii. 15	31. xii. 15	—	—	—	—	—	—	2	8	8	7	2	9	2	8	11	5	4	7	9
10	26. xii. 15	1. i. 16	—	—	—	—	2	3	1	—	3	2	6	3	3	1	—	—	—	—	—
11	28. xii. 15	2. i. 16	—	—	—	—	4	11	4	4	7	7	6	8	5	7	7	12	7	7	2
12	28. xii. 15	3. i. 16	—	—	—	—	—	1	5	4	3	9	3	1	1	1	—	1	—	—	—
13	30. xii. 15	4. i. 16	—	—	—	—	1	10	—	7	9	5	8	2	4	7	6	2	2	—	—
14	1. i. 16	5. i. 16	—	—	—	—	—	5	5	5	8	9	4	4	4	7	8	4	4	3	—
15	6. i. 16	6. i. 16	—	—	—	—	—	1	1	3	5	2	6	5	7	5	7	9	6	4	6
16	10. i. 16	10. i. 16	—	—	—	—	—	—	1	2	7	4	7	8	8	6	5	9	9	9	4
17	13. i. 16	13. i. 16	—	—	—	—	—	1	3	7	5	5	7	7	5	6	7	10	4	8	2
18	15. i. 16	15. i. 16	—	—	—	—	—	—	2	5	7	6	6	7	13	7	10	8	1	1	—
19	19. i. 16	19. i. 16	—	—	—	—	—	—	—	5	3	8	8	12	1	9	12	6	7	**3**	**1**
20	20. i. 16	20. i. 16	—	—	—	—	1	1	6	3	7	8	7	5	7	9	4	—	—	—	—
21	22. i. 16	22. i. 16	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

Figures in heavy type refer to ca

rticulars of the hatching of the eggs.

particular date. It was found impracticable to keep the eggs laid on different days separate from each
from which the number of days has been reckoned is the date on which the ♂ was placed with the ♀.

ed with the ♀									Total of eggs laid per ♀	Total of eggs hatched	Percentage of possible fertile eggs	Remarks
27th	28th	29th	30th	31st	32nd	33rd	34th	35th				
—	—	—	—	—	—	—	—	—	118	60	51 %	Ability to fertilize eggs retained for *less* than 20 days.
—	—	—	—	—	—	—	—	—	150	59	39 %	,, ,, ,, ,, 24 ,,
7	5	1	—	1	—	—	—	—	166	97	59 %	,, ,, ,, *more* ,, 19 ,,
—	—	—	—	—	—	—	—	—	172	56	33 %	,, ,, ,, *less* ,, 19 ,,
—	—	—	—	—	—	—	—	—	7	0	nil	It is doubtful if any pairing took place.
—	—	—	—	—	—	—	—	—	95	57	63 %	Ability to fertilize eggs retained for *at least* 10 ,,
—	—	—	—	—	—	—	—	—	123	65	57 %	,, ,, ,, *less* than 21 ,,
—	—	—	—	—	—	—	—	—	70	0	nil	It is doubtful if any pairing took place.
3	2	4	5	5	4	—	—	1	159	115	75 %	Ability to fertilize eggs retained for *at least* 20 ,,
—	—	—	—	—	—	—	—	—	102	24	28 %	,, ,, ,, ,, 6 ,,
—	—	1	—	—	—	—	—	—	180	99	60 %	,, ,, ,, ,, 18 ,,
—	—	—	—	—	—	—	—	—	55	29	66 %	,, ,, ,, ,, 7 ,,
—	—	—	—	—	—	—	—	—	154	63	46 %	,, ,, ,, *not more* than 18 ,,
—	—	—	—	—	—	—	—	—	181	70	41 %	,, ,, ,, ,, 20 ,,
3	10	1	1	4	1	—	—	—	148	96	65 %	,, ,, ,, *over* 16 ,,
5	1	1	—	—	—	—	—	—	137	88	64 %	,, ,, ,, ,, 15 ,,
—	—	—	—	—	—	—	—	—	141	77	55 %	,, ,, ,, *not more* than 19 ,,
—	—	—	—	—	—	—	—	—	152	73	48 %	,, ,, ,, ,, 17 ,,
1	—	—	—	—	—	—	—	—	115	77	67 %	,, ,, ,, *about* 15 ,,
—	—	—	—	—	—	—	—	—	117	58	50 %	,, ,, ,, *not more* than 12 ,,
—	—	—	—	—	—	—	—	—	110	0	nil	It is doubtful if any pairing took place with this ♂, see entries under Table V.

ch eggs laid in the second box hatched.

TABLE V.

Pediculus humanus (vestimenti). *Particulars of hatching of the eggs laid after the second ♂ had been placed with ♀♀.*

Reference number of ♀	Day counting from maturity of the ♀ when the second ♂ was introduced	Days counting from the date on which the second ♂ was placed with the ♀ 10th	11th	12th	13th	14th	15th	16th	17th	18th	19th	20th	21st	22nd	23rd	24th	25th	26th	27th	Total of eggs laid per ♀	Total of eggs hatched	Percentage fertile	Remarks
3	35th	—	2	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	13	2	15 %	Died 38th day.
9	33rd	—	3	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	4	3	75 %	,, 35th ,, whilst paired.
13	31st	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	0	0	,, 33rd ,, eggs infertile.
14	32nd	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	56	0	0	,, 41st ,, ,, ,,
15	28th	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	38	0	0	,, 37th ,, ,, ,,
16	25th	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	80	0	0	Placed with a third ♂ on 37th day and fertile eggs laid subsequently, see Table VI.
17	25th	—	—	8	6	5	5	7	6	6	4	3	1	—	—	—	—	—	—	108	51	47 %	Died 44th day.
18	24th	—	6	10	6	6	8	6	6	7	8	3	8	5	6	4	3	—	—	130	92	71 %	Placed with a third ♂ on 42nd day, see Table VI.
19	21st	—	3	7	6	6	7	2	3	5	1	5	—	—	—	—	—	—	—	58	45	78 %	Died 31st day.
20	21st	—	4	5	9	6	4	7	6	8	4	8	6	4	5	3	5	1	2	108	87	81 %	,, 39th ,,
21	19th	—	1	5	7	7	6	3	5	3	3	7	5	5	4	5	3	4	—	94	73	78 %	,, 33rd ,,

TABLE VI.

Pediculus humanus (vestimenti). *Particulars of hatching of the eggs after the third ♂ had been placed with ♀♀.*

Reference number of ♀	Days counting from maturity of the ♀ when the third ♂ was introduced	Days counting from the date on which the third ♂ was placed with the ♀ 10th	11th	12th	13th	14th	15th	Total of eggs laid per ♀	Total of eggs hatched	Percentage fertile	Remarks
16	37th	—	—	—	2	—	1	4	3	75 %	Died 39th day.
18	42nd	—	—	—	—	—	—	13	0	0	,, 46th ,, eggs infertile.

TABLE VII.

Pediculus humanus (vestimenti). *Summary of Tables I to VI.*

Reference number of ♀	Length of life in days	Number of days between maturity and pairing	Number of days between maturity and oviposition	Number of eggs laid before the introduction of a ♂	Number of eggs laid, pairing No. 1	Approximate period of fertility in days	Percentage of fertile eggs	Number of eggs laid, pairing No. 2	Approximate period of fertility in days	Percentage of fertile eggs	Number of eggs laid, pairing No. 3	Approximate period of fertility	Percentage of fertile eggs	Total number of eggs laid	Average number laid each day	Total number of eggs hatched	Percentage of *possibly fertile* eggs which hatched
1	23	1	1	3	118	less than 20	51 %	—	—	—	—	—	—	118	5·3	60	51 %
2	34	no choice	4	—	150	,, ,, 24	39 %	—	—	—	—	—	—	150	4·4	59	59 %
3	38	,,	2	2	166	more than 19	59 %	13	—	15 %	—	—	—	179	4·7	99	56 %
4	32	,,	2	1	172	less than 19	33 %	—	—	—	—	—	—	172	5·4	56	33 %
5	11	,,	2	6	7	infertile	nil	—	—	—	—	—	—	7	·6	nil	nil
6	19 (lost)	,,	3	5	95	at least 10	63 %	—	—	—	—	—	—	95	5·0	57	63 %
7	32	,,	3	10	123	less than 21	57 %	—	—	—	—	—	—	123	3·9	65	57 %
8	23 (killed)	,,	3	7	70	infertile	nil	—	—	—	—	—	—	70	3·0	—	nil
9	35	,,	4	5	159	at least 20	75 %	4	—	75 %	—	—	—	163	5·0	118	75 %
10	29	,,	2	15	102	,, ,, 6	28 %	—	—	—	—	—	—	102	3·5	24	28 %
11	30	,,	2	14	180	,, ,, 18	60 %	—	—	—	—	—	—	180	6·0	99	60 %
12	19	,,	3	11	55	,, ,, 7	66 %	—	—	—	—	—	—	55	2·9	29	66 %
13	33	,,	2	13	154	not more than 18	46 %	5	infertile	nil	—	—	—	159	4·8	63	43 %
14	41	,,	1	12	181	,, ,, 20	41 %	56	,,	,,	—	—	—	237	5·8	70	31 %
15	37	less than 1	3	—	148	over 16	65 %	38	,,	,,	—	—	—	186	5·0	96	52 %
16	39	,, ,, 1	3	—	137	,, 15	64 %	80	,,	,,	4	1 day	75 %	221	5·6	91	41 %
17	44	doubtful	2	—	141	not more than 19	55 %	108	at least 8	47 %	—	—	—	249	5·7	128	51 %
18	46	,,	3	—	152	,, ,, 17	48 %	130	,, ,, 13	71 %	13	infertile	nil	295	6·4	165	56 %
19	31	less than 1	3	—	115	about 15	67 %	58	,, ,, 7	78 %	—	—	—	173	5·8	122	71 %
20	39	doubtful	2	—	117	not more than 12	50 %	108	,, ,, 13	81 %	—	—	—	225	5·8	145	64 %
21	33	1	2	—	110	infertile	nil	94	,, ,, 11	78 %	—	—	—	204	6·2	73	36 %

Life of ♂ 32 days. Longest ♀ life 46 days. Average ♀ life 34 days. Number of ♀♀ fertilized by one ♂, 18. Eggs laid by unfertilized ♀♀ did not hatch. The period of fertility lasts about 12 to 21 days. The number of eggs fertilized during the period of fertility probably depends on the rate of egg laying, the highest record was 115 by ♀ No. 9. The egg laying capacity of normal ♀♀ is certainly conditioned by food (see Table VIII) and by temperature as pointed out by Sikora (1915). In the above experiment (omitting ♀ No. 5) the numbers range from 55 to 295 with an average of 177 and a daily average of 5·1.

TABLE VIII.

The influence of food supply upon (a) the number of eggs laid and (b) their fertility.

Method. A number of lice in the larval stage were reared until adult. 5 ♂♂ and 5 ♀♀ were then placed in each of two boxes. Those in box B were fed at night only, period for feeding available about 7 hours. In box A additional feeding time, 2 or 3 hours, was given by day. Boxes kept in a breast pocket by day. (A false start was made owing to a ♀ in one of the boxes escaping during the counting of the eggs; as the escape was not noticed until the 10th day a fresh start was made on the 11th day with four pairs in each box.)

Box A.

	Number of eggs laid each day. Figures at top indicate days in the life of the ♀							Number of eggs hatching, each day's laying treated as a separate batch						
	12	13	14 15	16	17	18	19	12	13	14 15	16	17	18	19
Number of eggs laid	32	25	66	34	35	34	30	30	23	62	32	30	30	26

Total 256 = a daily average of 8 per ♀ Total 233

Percentage hatching = 91 %.

Box B.

	Number of eggs laid each day. Figures at top indicate days in the life of the ♀							Number of eggs hatching, each day's laying treated as a separate batch						
	12	13	14 15	16	17	18	19	12	13	14 15	16	17	18	19
Number of eggs laid	28	17	49	30	22	28	27	28	17	49	29	21	25	26

Total 201 = a daily average of 6·3 per ♀ Total 195

Percentage hatching = 97 %.

Reverse order of feeding.

On the 20th and 21st days the insects in both boxes were treated alike as regards feeding (at night only) and no records of egg laying were made. On the 22nd and following days the insects were fed in the reverse order; A one period of about 7 hours at night, B two periods 7 hours at night and 2 or 3 hours during the day. On the 26th day a ♀ in box A died and one was also removed from box B. On the 28th day the insects in box B were not fed during the day and both boxes were removed from pocket during that day.

Box A.

	Number of eggs laid each day. Figures at top indicate days in the life of the ♀							Number of eggs hatching, each day's laying treated as a separate batch						
	23	24	25	26	27	28 29	30	23	24	25	26	27	28 29	30
Number of eggs laid	18	18	15	18	17	28	14	17	17	13	16	14	28	12

Total 128 = a daily average of 4·5 per ♀ Total 117

Percentage hatching = 91 %.

Box B.

	Number of eggs laid each day. Figures at top indicate days in the life of the ♀							Number of eggs hatching, each day's laying treated as a separate batch						
	23	24	25	26	27	28 29	30	23	24	25	26	27	28 29	30
Number of eggs laid	32	30	28	25	23	28	21	31	30	24	23	20	27	21

Total 187 = a daily average of 6·6 per ♀ Total 176

Percentage hatching = 94 %.

TABLE IX.

Hatching of eggs of Pediculus humanus *when kept at a constant temperature.*

The eggs laid by 10 pairs of newly-matured lice were taken from the boxes in which they were laid (on cloth) each day and kept as separate batches in a humid atmosphere at 30° C.

Number of days counting from the approximate date on which the insects matured	Total number of eggs laid	Record of hatching Number of days reckoning from the date of laying												Total number of eggs which hatched	Percentage of eggs which hatched
		1	2	3	4	5	6	7	8	9	10	11	12		
1															
2	29	—	—	—	—	—	—	4	12	5	—	—	—	21	72 %
3	60	—	—	—	—	—	—	3	38	6	—	—	—	47	78 %
4	68	—	—	—	—	—	—	2	48	15	1	—	—	66	94 %
5	71	—	—	—	—	—	—	—	24	18	16	—	—	58	82 %
6	84	—	—	—	—	—	—	—	56	13	9	—	—	78	93 %
7 8	120	—	—	—	—	—	—	4	52	45	13	—	—	114	95 %
9	61	—	—	—	—	—	—	—	26	26	—	—	—	52	85 %
10	71	—	—	—	—	—	—	—	21	40	5	—	—	66	93 %
11	62	—	—	—	—	—	—	—	24	24	12	—	—	60	97 %
12	60	—	—	—	—	—	—	—	34	18	6	—	—	58	97 %
13	42	—	—	—	—	—	—	2	29	8	1	—	—	40	95 %
14 15	115	—	—	—	—	—	—	9	68	29	5	—	—	111	97 %
16	64	—	—	—	—	—	—	—	15	36	10	—	—	61	95 %
17	57	—	—	—	—	—	—	—	14	32	4	1	—	51	89 %
18	62	—	—	—	—	—	—	—	20	32	2	1	—	55	89 %
19	57	—	—	—	—	—	—	—	13	16	22	1	—	52	91 %
20 21 22	No record														
23	50	—	—	—	—	—	—	1	40	7	—	—	—	48	96 %
24	48	—	—	—	—	—	—	—	30	16	1	—	—	47	98 %
25	43	—	—	—	—	—	—	—	24	13	—	—	—	37	86 %
26	43	—	—	—	—	—	—	1	25	13	—	—	—	39	91 %
27	40	—	—	—	—	—	—	—	27	7	—	—	—	34	85 %
28 29	56	—	—	—	—	—	—	6	45	4	—	—	—	55	98 %
30	35	—	—	—	—	—	—	4	29	—	—	—	—	33	94 %
	1398							36	714	423	107	3		1283	92 %

Summary

3 %	hatched on the	7th	day
56 %	,, ,,	8th	,,
33 %	,, ,,	9th	,,
8 %	,, ,,	10th	,,
0·2 %	,, ,,	11th	,,

TABLE X. *Fertility of* Pediculus capit

Insects in the second larval skin were taken from a stock box and reared until maturity in separate box Opportunity for feeding was given each night (approximately 7 hours). Owing to the early death of the indicated by the figures 1, 2, 3, 4 in the first column. ♂ No. 1 matured on the 5th January and died on on the 25th January and died on the 9th February, life 15 days. The last ♂ No. 4 matured on the 9th Februa capacity of an unfertilized ♀. Eggs laid before the introduction of a ♂ are italicised. The boxes contain

Reference number of ♂	Reference number of ♀	Date when ♀ matured	Date when the ♂ was added	Copulation observed	Date of removal of the ♂	Days counting from the maturity of the ♀ to that on which the e																	
						1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th	16th	17th	19th
No. 2	1	4. I. 16	17. I. 16	—	—	–	*1*	*2*	*3*	*2*	*5*	*2*	*4*	*2*	*3*	*2*	*2*	*4*	2	4	1	1	–
No. 1	2	5. I. 16	6. I. 16	—	9. I. 16	–	–	2	3	3	2	6	3	2	6	4	2	5	5	8	1	3	7
No. 1	3	9. I. 16	9. I. 16	—	died 12. I. 16	–	2	2	5	6	3	5	5	5	7	4	1	4	3	6	1	4	7
No. 2	4	13. I. 16	13. I. 16	—	16. I. 16	–	–	3	2	3	3	4	2	3	4	5	2	3	8	7	4	–	1
No. 2	5	16. I. 16	16. I. 16	—	died 17. I. 16	–	–	3	1	5	2	5	2	5	2	–	–	–	–	–	–	–	–
No. 3	6	27. I. 16	27. I. 16	—	31. I. 16	–	–	2	3	3	5	7	5	4	6	7	4	4	6	3	6	–	1
No. 3	7	31. I. 16	31. I. 16	—	5. II. 16	–	3	3	4	4	5	6	3	5	5	8	3	–	2	4	8	8	7
No. 3	8	5. II. 16	5. II. 16	—	8. II. 16	–	3	4	5	4	6	4	2	9	4	8	5	4	7	7	8	7	7
No. 3	9	8. II. 16	8. II. 16	—	died 9. II. 16	–	1	6	4	2	7	2	6	5	4	4	4	5	6	4	3	4	5
No. 4	10	8. II. 16	10. II. 16	—	11. II. 16	–	–	3	3	3	4	4	4	4	4	7	2	4	5	4	3	5	3
No. 4	11	11. II. 16	11. II. 16	—	12. II. 16	–	4	4	5	5	4	8	7	6	3	6	3	5	5	4	5	7	2
No. 4	12	12. II. 16	12. II. 16 15. II. 16	—	13. II. 16 16. II. 16	–	–	1	1	2	3	4	4	2	4	5	3	4	3	–	–	–	–
No. 4	13	13. II 16	14. II. 16	—	15. II. 16	–	–	3	3	2	5	4	6	1	3	4	4	5	5	–	4	2	6
No. 4	14	16. II. 16	16. II. 16	—	18. II. 16	–	–	3	3	2	5	4	4	5	3	3	6	7	3	4	6	6	2
No. 4	15	16. II. 16	18. II. 16	—	19. II. 16	–	*3*	3	7	1	3	6	3	7	3	5	1	7	4	5	3	–	5
No. 4	16	19. II. 16	19. II. 16 25. II. 16	20. II. 16	21. II. 16 26. II. 16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
No. 4	17	19. II. 16	21. II. 16	—	23. II. 16	–	–	–	2	3	4	4	4	–	5	3	6	5	3	2	7	1	6
No. 4	18	23. II. 16	23. II. 16	—	25. II. 16	–	–	5	1	5	4	4	2	2	2	6	6	5	6	5	–	7	3
No. 4	19	26. II. 16	26. II. 16	—	1. III. 16	–	2	4	4	–	1	–	–	–	–	–	–	–	–	–	–	–	–
No. 4	20	1. III. 16	1. III. 16	—	6. III. 16	–	1	3	3	3	3	2	6	5	2	3	–	–	–	–	–	–	–
No. 4	7	—	6. III. 16	7. III. 16	8. III. 16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
No. 4	9	—	8. III. 16	—	9. III. 16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
No. 4	10		9. III. 16	—	died 10. III. 16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Note. The figures in heavy type indicate that these eggs were kept in separ *articulars of pairing and egg laying.*

he ♀♀ were kept and fed in the boxes in which they matured, the ♂♂ being placed with them in rotation.
ie first three attempts were of doubtful value and a fourth was initiated with better success. These ♂♂ are
2th, life 7 days. ♂ No. 2 matured on the 12th January and died on the 24th, life 12 days. ♂ No. 3 matured
nd died on the 10th March, life 30 days. ♀ No. 1 was purposely kept unpaired for 12 days to test the egg laying
ie ♀♀ and eggs were carried in a waistcoat pocket during the day, at night they were against the body.

ere laid. The number of eggs laid is indicated by the figure below																	Total eggs laid per ♀	Remarks
21st	22nd	23rd	24th	25th	26th	27th	28th	29th	30th	31st	32nd	33rd	34th	35th	36th	37th		
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	40	Died on the 17th day.
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	62	,, ,, 22nd ,,
5	–	2	6	**3**	**3**	**3**	**3**	**3**	**3**	**3**	–	–	–	–	–	–	109	Placed with another ♂ on the 32nd day, see entries in Table XII.
4	**4**	**3**	**5**	**3**	**2**	–	–	–	–	–	–	–	–	–	–	–	81	Placed with another ♂ on the 28th day, see entries in Table XII.
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	25	Died on the 11th day.
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	66	,, ,, 20th ,,
6	6	5	4	4	6	–	4	4	4	5	3	2	5	–	2	–	138	Placed with ♂ No. 4 on the 36th day, see entries below.
–	4	–	4	5	–	–	–	–	–	–	–	–	–	–	–	–	107	Died on the 26th day.
5	4	3	2	4	–	3	–	7	–	–	–	–	–	–	–	–	101	Placed with ♂ No. 4 on the 30th day, see entries below.
5	6	–	4	6	5	–	–	6	3	–	–	–	–	–	–	–	102	,, ,, ,, 31st ,, ,, ,,
3	4	4	8	7	7	–	–	–	–	–	–	–	–	–	–	–	120	Died on the 29th day.
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	36	Accidentally killed on the 14th day.
3	4	3	–	5	1	6	–	–	–	–	–	–	–	–	–	–	82	Placed with another ♂ on the 28th day see further entries in Table XII.
7	4	3	2	3	–	–	–	–	–	–	–	–	–	–	–	–	85	,, ,, ,, 26th ,,
3	4	7	3	3	3	–	–	–	–	–	–	–	–	–	–	–	92	,, ,, ,, 27th ,,
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	0	Died on 15th day, presumably malformed as she developed no eggs.
2	6	5	3	1	–	–	–	–	–	–	–	–	–	–	–	–	77	Died on the 25th day.
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	65	Accidentally killed on the 20th day.
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	11	Died on the 14th day.
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	31	Died on the 12th day. A stoppage of the oviduct was probably responsible for the death, her body became excessively swollen with eggs which she did not lay.
–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	3	Died on the 38th day.
–	–	–	–	–	–	–	–	–	–	–	3	1	2	3	–	–	9	,, ,, 36th ,,
–	–	–	–	–	–	–	–	–	–	–	6	2	–	–	–	–	8	,, ,, 34th ,,

oxes in order to test if the ♀♀ still retained the power to fertilize.

TABLE XI.

Pediculus humanus (vestimenti). *Hatching of eggs at different temperatures.*

A large mass of eggs laid on a strip of cloth was taken from a stock box and divided into three portions, one of which was submitted to each of the following conditions: (*a*) in a room, the air of which was very dry owing to the central heating of the building at 15·6° to 18·4° C., (*b*) in a humid incubator at 24·5° C. constant, (*c*) in a dry air incubator at 36·1° C. constant.

	Day after removal from the stock box on which hatching occurred																								
Temperature	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th	11th	12th	13th	14th	15th	16th	17th	18th	19th	20th	21st	22nd	23rd	24th	Totals
at 15·6° to 18·4° C.	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	nil
at 24·5° C.	—	—	—	3	1	—	—	—	—	2	—	—	—	—	4	2	1	1	—	1	—	—	1	—	16
at 36·1° C.	—	—	—	21	32	53	25	12	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	143

NOTE. This table only shows the comparative time of hatching, the actual period would have been a few days longer. Prior to the removal of eggs from the stock box it would have contained some in an advanced stage of development. Probably the eggs on the verge of hatching would in some cases be killed or delayed by the sudden alteration in the conditions as the stage immediately preceding hatching seems to be a critical one.

TABLE XII.

Fertility of Pediculus capitis. *Particulars of egg laying after a second ♂ was placed with the ♀♀.*

Reference number of ♀	Day counting from maturity of ♀ when the second ♂ was introduced	Date on which the second ♂ was introduced	Date of copulation when observed	Date when the ♂ was removed	Day, counting from maturity of the ♀, on which eggs were laid after the introduction of the second ♂												Total eggs laid per ♀	Remarks
					26th	27th	28th	29th	30th	31st	32nd	33rd	34th	35th	36th	37th		
4	27th	8. II. 16	—	9. II. 16	—	—	—	3	1	—	—	—	—	—	—	—	4	Died 31st day.
3	32nd	9. II. 16	—	10. II. 16	—	—	—	—	—	—	—	2	1	—	—	—	3	,, 34th ,,
13	28th	10. III. 16	—	11. III. 16	—	—	—	2	3	2	2	2	2	—	—	—	13	,, 35th ,,
14	25th	10. III. 16	14. III. 16	17. III. 16	4	4	3	4	3	—	—	—	—	—	—	—	18	,, 31st ,,
15	27th	13. III. 16	15. III. 16	17. III. 16	—	—	—	2	3	5	2	5	3	3	2	—	25	,, 37th ,,

TABLE XIII.

Fertility of Pediculus capitis. *Particulars of the hatching of the eggs.*

NOTE. There is no necessary relation between the lice emerging on any particular day and the eggs laid on any particular date. It was found impracticable to keep the eggs laid on different days separate, they were allowed to accumulate in the box with the laying ♀ until she was transferred to a new box. The zero date from which the number of days has been reckoned is the date on which the ♂ was placed with the ♀.

Reference number of ♀	Date when the ♀ matured	Date when the ♂ was added	Days counting from the date on which the ♂ was placed with the ♀																					Total no. of eggs laid per ♀	Total no. which hatched	Percentage of possible fertile eggs	Remarks
			8th	9th	10th	11th	12th	13th	14th	15th	16th	17th	18th	19th	20th	21st	22nd	23rd	24th	25th	26th	27th	29th				
1	4. I. 16	17. I 16	–	–	–	–	2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	40	2	25 %	Eggs laid prior to the introduction of a ♂ all failed to develop.
2	5. I. 16	6. I. 16	–	–	–	–	1	–	–	4	2	3	2	4	6	1	–	2	1	2	–	3	–	62	31	50 %	Ability to fertilize eggs retained for at least 7 days.
3	9. I. 16	9. I. 16	–	–	–	–	–	1	1	2	3	1	1	2	1	1	1	1	1	–	–	–	–	109	16	15 %	,, ,, ,, ,, 3 ,,
4	13. I. 16	13. I. 16	–	–	–	–	–	–	1	2	4	1	1	1	5	–	5	7	5	3	4	5	–	81	44	54 %	,, ,, ,, ,, 12 ,,
5	16. I. 16	16. I. 16	–	–	–	–	4	1	3	2	2	3	2	2	2	–	–	–	–	–	–	–	–	25	21	84 %	,, ,, ,, ,, 8 ,,
6	27. I. 16	27. I. 16	–	–	–	–	–	1	4	–	4	6	–	8	4	6	3	3	4	1	–	–	–	66	44	67 %	,, ,, ,, ,, 8 ,,
7	31. I. 16	31. I. 16	–	–	–	–	1	4	3	5	2	4	6	3	5	5	5	3	5	3	3	4	–	138	61	44 %	,, ,, ,, ,, 11 ,,
8	5. II. 16	5. II. 16	–	2	–	1	1	6	3	4	3	5	8	4	4	6	3	3	8	7	–	2	–	107	70	65 %	,, ,, ,, ,, 12 ,,
9	8. II. 16	8. II. 16	–	–	–	–	2	1	6	3	2	5	4	1	6	6	3	5	–	1	–	–	–	101	45	45 %	,, ,, ,, ,, 11 ,,
10	8. II. 16	10 II. 16	–	–	–	–	–	4	3	1	6	3	3	2	6	1	3	1	4	1	–	1	–	102	39	38 %	,, ,, ,, ,, 10 ,,
11	11. II. 16	11. II. 16	–	–	–	–	–	2	3	3	2	4	3	5	7	4	1	1	–	–	–	–	–	120	35	29 %	,, ,, ,, ,, 7 ,,
12	12. II. 16	12. II. 16 15. II. 16	–	–	–	–	–	–	–	2	1	4	2	3	4	1	4	–	3	1	–	–	–	36	25	69 %	,, ,, ,, about 7 ,,
13	13. II. 16	14. II. 16	–	–	–	1	2	1	1	2	10	2	2	2	3	3	1	3	2	1	–	–	–	82	36	44 %	,, ,, ,, at least 11 ,,
14	16. II. 16	16. II. 16	–	–	–	–	–	–	1	–	2	1	1	2	2	1	4	2	2	3	1	1	2	85	25	29 %	,, ,, ,, ,, 7 ,,
15	16. II. 16	18. II. 16	–	–	–	3	4	2	2	1	3	5	2	2	4	3	3	4	3	3	1	1	–	92	46	50 %	,, ,, ,, ,, 10 ,,
16	19. II. 16	19. II. 16 25. II. 16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	nil	nil	—	This female was presumably malformed, her ovaries failed to develop though she lived 15 days, fed vigorously and was observed to pair.
17	19. II. 16	21. II. 16	–	–	–	1	4	1	2	3	5	1	1	1	3	1	4	2	2	2	1	1	–	77	35	45 %	Ability to fertilize eggs retained for at least 9 days.
18	23. II. 16	23. II. 16	–	–	–	–	1	2	–	3	5	3	1	1	6	2	3	7	5	2	1	3	1	65	46	71 %	,, ,, ,, ,, 12 ,,
19	26. II. 16	26. II. 16	–	–	–	–	–	3	3	3	1	–	–	–	–	–	–	–	–	–	–	–	–	11	10	91 %	
20	1. III. 16	1. III. 16	–	–	–	–	–	–	1	1	1	–	3	1	–	–	4	–	–	–	–	–	–	31	11	35 %	
7		6. III. 16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	3	nil		
9		8. III. 16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	9	nil		
10		9. III. 16	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	8	nil		

TABLE XIV.

Fertility of Pediculus capitis. *Particulars of hatching after a second ♂ was placed with the ♀♀.*

Reference no. of ♀	Day, counting from maturity of the ♀, when the second ♂ was introduced	Days counting from the date on which the second ♂ was placed with the ♀						Total eggs laid per ♀	Total of eggs hatched	Percentage fertile	Remarks
		11th	12th	13th	14th	15th	16th				
4	27th	1	—	—	—	—	—	4	1	25 %	died 31st day
3	32nd	1	—	—	—	—	—	3	1	33 %	,, 34th ,,
13	28th	—	—	—	—	—	—	13	nil	—	,, 35th ,,
14	25th	—	—	—	—	—	—	18	nil	—	,, 31st ,,
15	27th	—	1	—	1	3	1	25	6	24 %	,, 37th ,, *

* Although no more eggs hatched the balance had mostly been fertilized, the larvae developed but died just prior to hatching.

TABLE XV.

Pediculus capitis. *Summary of Tables X, XII—XIV.*

Reference number of ♀	Reference number of ♂	Number of days between maturity and pairing	Number of days between maturity and oviposition	Number of eggs laid before the introduction of a ♂	Number of eggs laid, pairing No. 1	Approximate period of fertility in days	Percentage of fertile eggs	Number of eggs laid, pairing No. 2	Percentage of fertile eggs	Total number of eggs laid per ♀	Average number laid each day	Total number of eggs hatched	Percentage of possible fertile eggs which hatched
1	2	no choice	2	32	40	1	25 %	—	—	40	2·5	2	25 %
2	1	doubtful	3	nil	62	at least 7	50 %	—	—	62	3·9	31	50 %
3	1	,,	2	,,	109	,, ,, 3	15 %	3	33 %	112	3·6	17	15 %
4	2	,,	3	,,	81	,, ,, 12	54 %	4	25 %	85	3·3	45	53 %
5	2	same day	3	,,	25	,, ,, 8	84 %	—	—	25	3·1	21	84 %
6	3	doubtful	3	,,	66	,, ,, 8	67 %	—	—	66	4·1	44	67 %
7	3	,,	2	,,	138	,, ,, 11	44 %	3	nil	141	4·0	61	43 %
8	3	,,	2	,,	107	,, ,, 12	65 %	—	—	107	4·7	70	65 %
9	3	same day	2	,,	101	,, ,, 11	45 %	9	nil	110	3·5	45	41 %
10	4	no choice	3	,,	102	,, ,, 10	38 %	8	nil	110	3·8	39	36 %
11	4	1	2	,,	120	,, ,, 7	29 %	—	—	120	4·4	35	29 %
12	4	probably 3	3	,,	36	about 7	69 %	—	—	36	3·0	25	69 %
13	4	no choice	3	,,	82	at least 11	44 %	13	nil	95	3·1	36	38 %
14	4	doubtful	3	,,	85	,, ,, 7	29 %	18	nil	103	3·8	25	24 %
15	4	,,	2	3	92	,, ,, 10	50 %	25	24 %	117	3·7	52	44 %
16	4	1	...	nil	nil	...	...	—	—	nil	nil	nil	nil
17	4	doubtful	4	,,	77	at least 9	45 %	—	—	77	3·7	35	45 %
18	4	,,	3	,,	65	,, ,, 12	71 %	—	—	65	3·8	46	71 %
19	4	,,	2	,,	11	3	91 %	—	—	11	2·2	10	91 %
20	4	,,	2	,,	31	at least 6	35 %	—	—	31	3·1	11	35 %

Longest ♂ life 30 days. Longest ♀ life 38 days. Average ♀ life 27 days. Number of ♀♀ fertilized by one ♂ 10. Eggs laid by unfertilized ♀♀ did not hatch. The period of fertility lasts about 7 to 12 days. The greatest number of eggs fertilized by a ♀ after separation from a ♂ was 70 (♀ No. 8). The egg laying record in the above experiment ranges between 25 and 141 with an average of 88 and a daily average of 3·7. It is probable that owing to the unnatural conditions of the experiment that these figures are too low as an estimate of the egg production of wild lice.

the real period of fertility exceeded that calculated, it being improbable that there would be no failures among the early laid eggs. As soon as the ♀♀ ceased to lay fertile eggs some of them were given the opportunity of a second pairing. Particulars of the egg laying subsequent to the introduction of a second ♂ are set out in Table II, and of the hatching of the eggs in Table V. It will be seen that a number of the ♀♀ regained fertility after a second pairing, while two were afforded the option of a third. One of these (No. 16) apparently missed the second opportunity, but availed herself of the third and laid a few fertile eggs; the other (No. 18) survived long enough to lay a few more eggs, but none of them hatched. Details are given in Tables III and VI[1].

Although the ♀♀ are stated to have no receptaculum seminis it will be seen that they are able to lay fertile eggs as long as 20 days after the removal of the ♂. This does not compare so unfavourably with fleas, in which the receptaculum is well developed. The ♀ of *Pulex irritans* requires to pair several times if all the eggs she is capable of laying are to be fertilized.

Table VII summarises the previous ones.

Table VIII shows the results of an experiment in differential feeding. Owing to the escape of a ♀ from Box A, it was necessary to remove one from Box B, and to discard observations made prior to this reduction in numbers. Consequently, the experiment covers the later, instead of the earlier, two-thirds of the insects' lives, as was intended. It is probably owing to this that there is a marked falling off in the average number of eggs laid during the reversed order of feeding, when the lice in Box B were fed twice a day and those in Box A only once. Nevertheless, the result shows clearly how dependent egg laying is on food supply—the fertility of the eggs remaining unaffected.

Hatching of eggs. Table IX shows details of the hatching of a number of eggs laid by 10 pairs of *P. humanus* from the date of their maturity until the 30th day. The eggs were placed in an incubator at a constant temperature of 31° C., the air being kept humid by a pan of water with slips of filter paper dipping into the water and hanging over the side of the pans. The high percentage hatching suggests that the conditions were not unfavourable. The variability shown in hatching may have been somewhat enhanced by the fact that the time of examination occasionally varied by three or four hours. It would be possible for the three records of hatching on the 11th day to be due to an earlier examination than usual on the previous day.

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[1] It is of course obvious that the percentage of eggs hatching in these Tables, as well as those in the parallel series for *P. capitis* Tables X and XII, has no real relation to the natural fertility of the eggs in the correct sense of the term; the figures are only inserted as a convenience.

Table XI gives the record of an experiment of the hatching of batches of eggs taken from a stock box and placed under varied conditions. None of the eggs hatched at room temperature, 15·6° to 18·4° C., but a few lice emerged from eggs kept at 24·5° C.

The widely distributed dates of hatching, in some instances after a long interval of time, of these eggs is in marked contrast to the uniformly rapid hatching of eggs kept at 36·1° C. and reminds us of the instance recorded by Warburton (1909), although the period of delay is far shorter.

Although the eggs were not counted, there is no doubt that many died when maintained at 24·5° C.; the batch submitted to cool conditions, 15·6° to 18·4° C., was smaller than the one placed at 36·1° C. but the number must have been upwards of 100.

Pediculus capitis. The attempt with this species to parallel the series of breeding experiments carried out with *P. humanus* were less successful. Possibly the former constituted a chance observation that it would be difficult to repeat without numerous failures, but more probably the conditions were less favourable for the head than for the body louse. There is, however, probably a real difference in the length of life and fecundity of the two insects even if this appears somewhat exaggerated in the protocol.

From Table X it will be seen that four ♂♂ were used in fertilizing the series of 20 ♀♀, the first three ♂♂ dying early in their career, but the fourth fertilized 10 ♀♀ and certainly paired once at least with a malformed ♀ that developed no eggs. A shortage in the supply of virgin ♀♀ was responsible for the fewer opportunities afforded to this ♂ than to the *P. humanus.* The fact that the ♂♂ in many instances were left longer with the ♀♀ than the one day which was usual in the parallel experiment with *P. humanus* probably accounts for there being no failures in fertilization. Similarly the periods during which the ♀♀ retain the power to lay fertile eggs may have been understated since they have been reckoned from the date upon which the male was removed.

Table XIII shows details regarding the hatching of the eggs recorded in Table X. Owing to the earlier loss of the power to lay fertile eggs in this species, the period is less clearly defined as regards its upper limit than in the case of *P. humanus,* but there is little doubt but that it is really shorter by about one-third.

Tables XII and XIV show the distribution in time of egg laying and hatching for the ♀♀ of the series which were afforded the opportunity of pairing with a second ♂.

Although the evidence obtained with regard to *P. capitis* is not so full as for *P. humanus*, it is sufficient to show that the habits of pairing, egg laying and fertilization are similar in both species.

HYBRIDIZATION.

An attempt was made to hybridize the two insects with a view to obtaining evidence bearing upon the debated question of their right to specific rank. It was found that when single pairs were confined in the same box, the ♂♂ of *capitis* and ♀♀ of *humanus*, or vice versa paired freely. In half the attempts, however, the ♀♀ of *P. capitis* were killed in the act of pairing or died within a day or two, presumably as the result of renewed attempts. It was found necessary, if any number of eggs was required, to remove the ♂ *P. humanus* after the pairing had been consummated. No such precaution was needed in the case of the reverse pairing, and although some of the ♂♂ of *P. capitis* used died early, they succeeded in fertilizing the eggs.

A noticeable feature of the pairing between *P. capitis* ♂ and *P. humanus* ♀ was the disparity of the sexes in the F. 1 generation of some of the crosses. The first trial gave 71 ♂♂ (= 74 %) against 25 ♀♀ (= 26 %), three specimens being killed in the nymph stage. There was considerable mortality in the egg state, which possibly accounted for the small number of ♀♀, although it affords no satisfactory explanation of the disparity of the sexes. No deaths were observed in the larval or nymph stage.

Pairing No. 2 of this cross gave 130 ♂♂ (= 86 %) and 22 ♀♀ (= 14 %), 44 of the ♂♂ had matured before the first ♀♀ developed.

Pairing No. 3 gave 51 ♂♂ (= 51 %) and 49 ♀♀ (= 49 %).

Pairing No. 4 gave 76 ♂♂ (= 68 %) and 35 ♀♀ (= 32 %).

In the F. 2 and F. 3 generations arising from cross pairing No. 1 the number of ♂♂ and ♀♀ appeared normal in all the boxes whether they contained single pairs or the eggs resulting from a number of individuals laid in a stock box. A considerable number of specimens were preserved without selection and an examination of these insects shows the following result:

F. 2 generation, 211 ♂♂ (= 54 %) and 181 ♀♀ (= 46 %).

F. 3 generation, 93 ♂♂ (= 46 %) and 110 ♀♀ (= 54 %) also 80 nymphs.

With the F. 1 generation of reverse cross, *P. humanus* ♂ and *P. capitis* ♀, the disparity was not so obvious.

The first trial failed owing to the death of the ♀ whilst pairing.

A second attempt was more successful and about 50 eggs were laid, the ♀ dying on the 17th day.

The F. 1 generation consisted of 27 ♂♂ (= 55 %) and 22 ♀♀ (= 45 %).

In the third trial the ♀ died after laying one egg.

A fourth attempt was made; two ♀♀ of *P. capitis* being confined with one ♂ of *P. humanus.* One of the ♀♀ died within a day or two and the other was left with the ♂, she laid about 30 eggs, but died within 10 or 12 days.

The F. 1 generation from this pairing consisted of 17 ♂♂ (= 71 %) and 7 ♀♀ (= 29 %).

One or two other attempts were made which resulted in the death of the ♀♀.

Of the subsequent generations reared from the F. 1 generation of the second pairing, a large number of specimens were reared and killed as they reached maturity. They consist of a portion, not the whole, of the offspring of two single pairings and a number of pairs left together in a stock box.

The specimens of the F. 2 generation are

143 ♂♂ (= 57 %), 109 ♀♀ (= 43 %) and 6 nymphs.

The specimens present of the F. 3 generation are

63 ♂♂ (= 50 %), 64 ♀♀ (= 50 %).

In size the specimens of the F. 1 and F. 2 generations of both crosses are generally intermediate between those of *P. humanus* and *P. capitis,* but in the F. 3 generation considerable disparity was noticeable—some very large ♂♂ and some very small ♀♀ being observed.

In the F. 2 generation, observations were made in regard to the egg laying habit, both hair (human) and cloth having been placed in boxes with a few selected pairs of hybrids of the F. 2 generation of the cross *P. capitis* ♂ and *P. humanus* ♀. I noticed that the ♀♀ laid on hair for choice, only a few eggs being attached to the cloth, but the lice showed the instinct to cluster their eggs, a few only being generally scattered along the hairs as is the case with eggs of *P. capitis*[1].

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[1] The F. 1 generation of this cross, resulting from a later pairing, show a marked preference for laying on cloth; out of four pairings three ♀♀ laid their eggs on cloth, while one selected hair and laid nearly all her eggs upon it. Unfortunately I was too busy to note the egg laying habit of the F. 1 generation of the earlier pairing.

The ♀♀ of the F. 2 generation of the cross, *P. humanus* ♂ and *P. capitis* ♀, laid their eggs on both cloth and hair, but they showed a preference for laying on hair; clustering was not so noticeable a feature as in the case of the ♀♀ of the reverse cross.

An examination of the eggs laid by these hybrids showed that on the whole they were clearly intermediate in size between those of the species. The eggs are, however, variable in size, both as regards those of the species and those of the hybrids, so that it was easy to match eggs of either *capitis* or *humanus* from the hybrid batch.

No signs of unhealthiness, mortality in moulting or shortening of life in the hybrid insects was observed; the usual life was 30 to 40 days. One pair of the F. 1 generation of the *P. capitis* ♂ and *P. humanus* ♀ cross lived 45 days and their fecundity appeared to be on a par with that of *P. humanus.* They throve just as well under the artificial conditions of rearing as did this species. Although *P. capitis* can be successfully reared in the boxes, the colonies do not show the same rapid and vigorous growth.

There was no noticeable increase of mortality in the eggs laid by the hybrid insects.

No attempts were made to carry the hybrid races beyond the F. 3 generation; they were then in all respects healthy and fertile, probably they could be continued indefinitely. The variability in size referred to above may, however, have been the beginning of a segregating process which would eventually result in the hybrid races being forced back to the specific norm.

SUMMARY.

General comparative note on the two species.

Pediculus humanus (*vestimenti*) is a larger, more robust and less active insect than *P. capitis,*—the ♀♀ having a relatively greater egg-carrying capacity than those of the head louse. The eggs are larger and the number laid (under the conditions of these experiments) is greater, while the habits associated with egg laying differ, although placing the ♀♀ of *humanus* under conditions applicable to *capitis* or vice versa may induce a considerable degree of uniformity. Cross pairings between the insects are easily brought about and the offspring are fertile *inter se.* Hybrid strains were maintained until the F. 3 generation and there seemed no reason, judging from breeding results, why such strains should not be continued indefinitely. Nevertheless the marked disparity in the sexes of the F. 1 generation of some of the crosses between *P. capitis* ♂ and *P. humanus* ♀ suggests that the parents are specifically distinct.

No such obvious disparity occurred between the sexes of the F. 2 and F. 3 hybrid generation, or of either of the pure stocks[1].

Habits. The body louse exhibits some of the habits of a gregarious animal especially during the moulting phases, also a preference for returning to the same spot for oviposition, which leads to the clustering of its eggs. These habits are shown, though in a less marked degree, by *P. capitis,* and it is possible therefore that they are to some extent the outcome of confinement. Pairing within both species took place at any time during day or night, and was very frequently observed after feeding. ♂♂ with but little food in their alimentary tract were, however, often seen in coitus. The period during which the insects remained paired was frequently observed to be over an hour, but no upper limit was defined.

A ♂ of *P. humanus* fertilized 18 out of 21 ♀♀ placed with him in succession. Four attempts with *P. capitis* were less successful; one ♂ fertilized ten ♀♀ and very possibly might have equalled the *P. humanus* record but for a scarcity of virgin ♀♀ while the experiment was in progress. The longest period during which a ♀ of *P. humanus* retained the power to lay fertile eggs in the absence of a ♂ was 20 days, usually it would seem to be from 16 to 18 days. In the case of *P. capitis* the period was shorter; 12 days being the longest ascertained period, while it was more usually from seven to eleven days.

The greatest number of eggs laid by any one ♀ of *P. humanus* was 295, an average of 6·4 per day—the daily average of a number of ♀♀ being 5·1. *P. capitis* ♀♀ showed a lower fecundity, the highest record being 141 with a daily average of 4—the general average being 3·7. These figures are probably exceeded under natural conditions. An experiment in differential feeding with *P. humanus* (Table VIII) shows clearly that fecundity is dependent on feeding. When extra feeding time over and above seven hours per day was given the average for four ♀♀ was eight per day. It is reasonable to suppose that the average for *P. capitis* would also be increased by unrestricted feeding.

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[1] Since the above went to press I have reared two broods of *P. humanus* from crosses between pale and dark forms of this species with a view to discovering if the melanic race shows Mendelian inheritance. In the case of a pairing between a pale ♂ and a dark ♀ there resulted 15 ♂♂ and 54 ♀♀, while from the reverse cross 102 ♂♂ and only 15 ♀♀ were bred. This result qualifies the above suggestion and necessitates further breeding experiments which are now in progress. (See p. 259, paper by Hindle.)

The fertility of the eggs laid was not affected by increased feeding. The greatest number of fertilized eggs laid by a ♀ *P. humanus* after the removal of the ♂ was 115 (♀ No. 9), with a ♀ showing a higher daily laying average this might well be exceeded. With *P. capitis* the parallel figure is 70 (♀ No. 9). The ♀♀ of both species, after arriving at maturity, started oviposition irrespective of their having paired or not, but eggs laid by virgin ♀♀ were invariably infertile.

Length of life. The life of the ♂ *P. humanus* used in the experiment recorded in Table I was 32 days; the longest ♀ life was 46 days, with an average of 34. For *P. capitis* the figures were: ♂ life 30 days; ♀ life 38 days, with an average of 27 days. Whether or not the average lives of the insects would be extended by unrestricted feeding is an open question.

The life of the hybrid insects was not noticeably shorter than that usual for *P. humanus,* and they seemed to thrive better than *P. capitis.*

Tests made with *unfed P. humanus* showed that the longest lives were at a medium temperature of 16° to 18° C., many of the insects living from three to four days, while two lived five and one lived seven days. At 24·5° C. all died within five days. At 36·1° C. all died within three days.

Newly-hatched larvae, unless fed, lived less than 24 hours at 36·1° C., and when kept in a box in the vest pocket they lived but little more than a day; none survived a second day.

Adults kept in a box in the side pocket of a coat lived five days without food; this was in March.

Moulting. 40 young lice were reared in a box carried in a vest pocket and particulars of their moulting recorded.

1st moult: 3 % moulted on the 3rd day; 42 % on the 4th and 55 % on the 5th day.

2nd moult: 15 % moulted on the 7th day; 72 % on the 8th and 13 % on the 9th day.

3rd moult: 5 % moulted on the 10th day; 3 % on the 11th, 55 % on the 12th, 32 % on the 13th day, while 5 % took 14 days to reach maturity.

The ♂♂ usually mature rather earlier than the ♀♀.

Cold. Active specimens of *P. humanus* survived two days at a temperature of − 2·3° C. to − 1·1° C., but none recovered after exposure to these conditions for a week.

Hatching of eggs. Table IX shows that under humid conditions at 31° C. 3 % of the 1300 eggs tested hatched on the 7th day; 56 % on the 8th; 33 % on the 9th; 8 % on the 10th and ·2 % later on the same day or on the 11th.

A test of batches of eggs taken from a stock box, some of which must have been laid several days previously, showed that none hatched at 15·6°–18·4° C., while at 24·5° C. there was considerable egg mortality, and the hatching period was spread over a longer period than usual, though not to the extent mentioned by Warburton (1909); at 36·1° C. hatching was spread over five days and the mortality was not excessive.

To give some idea of the possible rate of multiplication of *P. humanus* we may estimate the egg period as 12 days and a further 12 days to the maturity of the ♀♀. Allowing an average of eight eggs per day, spread over a fertility period of 40 days, we find that, during her life, a single ♀ may have 4160 offspring.

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(Chairman—Lieutenant-Colonel C. J. MARTIN, R.A.M.C., F.R.S.)

The Louse Problem.

By A. W. BACOT.[1]

OF the three kinds of Anoplura parasitic upon man only one, *Pediculus humanus* (the clothes louse), is of sufficient importance as a disease transmitter to cause serious anxiety on the part of military authorities for its control. *Phthirius pubis* (the crab louse), a small, readily distinguished insect of very specialized form is restricted to limited hair-clad areas of the body. It is disreputable rather than dangerous.

Pediculus capitis (the head louse) is so closely related to the body louse that it is only grudgingly, if at all, allowed specific rank apart from the latter. Many authorities are disposed to treat the two insects as more or less stable races, separated by structural details of minor importance. The divergence of habits between *Pediculus capitis* and *Pediculus humanus* in relation to their host would seem, however, to create a position which justifies distinctive nomenclature, as well as separate consideration from the practical point of view.

In popular estimation the presence of any of these three insects affords proof of personal uncleanliness, lice being still very generally conceived of as breeding in, if they do not actually feed on, dirt. The association with dirt is really adventitious, arising from the fact that the personal neglect, either enforced or voluntary, which is essential for any permanence of the infection, is almost invariably accompanied by a lack of soap, water, and clean garments, whereas it would of course be an easy matter to free oneself from lice and yet avoid washing.

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[1] Entomologist to the Lister Institute.

As regards food the extreme specialization of mouth parts restricts all three insects to an exclusive diet of blood, obtained under what are probably sterile conditions, so far as any matter extraneous to fluids circulating within the body is concerned.

With *Phthirius pubis* I do not propose to deal and with *Pediculus capitis* only in so far as its distinctive identity from *Pediculus humanus* is concerned.

IMMATURE PERIOD.

Lice belong to the group of insects having an incomplete metamorphosis. Between the larval, nymph and adult stage there is no break in general resemblance, mode of life or method of feeding. The only distinguishing points of any practical importance between immature and adult insects is the smaller size and consequent ease of hiding of the young, together with their ability, during the moulting phases, to resist the action of heat and insecticides for a somewhat longer period. As a set-off to these disadvantages, from their host's point of view, we have only the fact that they extract less blood.

Between the period of emergence from the egg and maturity, both *Pediculus capitis* and *Pediculus humanus* shed their skins thrice; the time occupied differs in relation to food supply, temperature and innate variability under reasonably favourable conditions; twelve days may be accepted as a fair average. The record of forty larvæ which emerged on the same day, were kept in a box in a vest pocket and afforded opportunities of feeding during seven out of each twenty-four hours, will give a fair idea of their constitutional variability.

FIRST MOULT.	SECOND MOULT.	THIRD MOULT.
3 per cent. on third day.	15 per cent. on seventh day.	5 per cent. on tenth day.
42 per cent. on fourth day.	72 per cent. on eighth day.	3 per cent. on eleventh day.
55 per cent. on fifth day.	13 per cent. on ninth day.	55 per cent. on twelfth day.
		32 per cent. on thirteenth day.
		5 per cent on fourteenth day.

PAIRING.

Pairing takes place at any time. It was frequently observed when the insects were examined after feeding, but the males on these occasions had sometimes little if any food in the alimentary canal. The period during which the insects remain together is generally considerable, many occasions of over an hour were observed. This is probably by no means the limit, but failing prolonged watching one cannot be certain that the union is a prolongation and not a repetition. As happens with the fleas, the male underlies the female, a position which renders it possible for both sexes to feed during the act of fertilization. The males apparently require some hours' interval between the final ecdysis and the act of coitus, but females, still quite soft and not fully expanded after their recent change from nymph to adult, are often seen paired.

EGG LAYING.

Food and temperature are the governing conditions in egg production. Feeding is essential, but laying may commence as early as the second or third day of adult life; by which time the female may have partaken of several meals and, apart from very exceptional circumstances, will have been fertilized. Virgin females, however, lay quite as freely as impregnated ones, only their eggs do not hatch.

Temperature approximating to that between the outer garments and the body is essential (75° F. to 93° F.). Sikora points out that it is probable that when day garments are removed and exposed to cool air (below 60° F.) at night, egg development will be stopped, although feeding may take place by day.

There is a cleavage in habits between the head and clothes louse in regard to the instinct and method of oviposition. Under normal conditions *Pediculus capitis* lays its eggs on hair, *Pediculus humanus* on textile material. In captivity either species can be induced to lay on hair or woven fabric if these conditions are properly adjusted. Tests in boxes lined with flannel and containing a small quantity of human hair resulted in the female of *Pediculus capitis* laying 80 per cent. on the hair and 20 per cent. on the flannel, while those of *Pediculus humanus* laid 20 per cent. on the hair and 80 per cent. on the flannel.

Individuals of both species were then reared from these eggs, which had been carefully segregated so that the following four lots of adults were obtained: *Pediculus humanus* reared from the eggs laid on hair; a second lot reared from the eggs laid on the flannel, and two batches of *Pediculus capitis*, one reared from the eggs laid on hair and the other from those laid on the flannel. Twelve males and twelve females of each of these four lots were placed in similar boxes, lined with flannel and containing a quantity of human hair, great care having been taken to get the quantity and arrangement of the hair similar in each box. The boxes were kept under the same conditions and the feeding periods and arrangements were as nearly as possible identical. After five days the eggs were counted and their position recorded with the following results:—

EGG-LAYING INSTINCTS OF *Pediculus capitis* AND *Pediculus humanus.*

Pediculus humanus bred from Eggs laid on Hair. 12 and 12.

359 eggs	On hair	38	= 11 per cent.
	On gauze cover of box	1	
	On flannel, side next box	37	= 10 ,,
	On flannel, exposed side	283	= 79 ,,

Eggs laid on hairs only when these came into contact with the flannel.

Pediculus humanus bred from Eggs laid on Flannel. 12 and 12.

344 eggs	On gauze cover of box	4	= 1 per cent.
	On flannel, side next box	55	= 16 ,,
	On flannel, exposed side	285	= 83 ,,

Pediculus capitis bred from Eggs laid on Hair. 12 and 12.

274 eggs	On gauze cover of box	1	
	On flannel, exposed side	14	= 5 per cent.
	On hair	259	= 95 ,,

Pediculus capitis bred from Eggs laid on Flannel. 12 and 12.

340 eggs	On gauze cover of box	4	= 1 per cent.
	On flannel	2	= ½ ,,
	On hairs close to flannel	2	= ½ ,,
	On hair	332	= 98 ,,

This result confirms the previous one as to the distinctiveness of the ovipositing instinct and clears away any doubt as to the possibility of the females being heterzygotes of the same species. The less fixed character of the instinct exhibited in the first trial being probably due to the individuals of *Pediculus capitis* being taken from a stock box lined with flannel, the central space of which consisted of a matted mass of hair, while in the case of *Pediculus humanus* the box was filled with a roll of flannel: under these circumstances it is not improbable that the instincts became dulled, and casual action resulted. Some care is necessary in arranging the boxes; these must not be too small and the amount of hair and its arrangement must not be such as to cause a mat of interlacing hairs at any one point, more especially near the top or bottom of the box. A previous experiment which I performed largely failed owing to a want of discretion on these points; the lice used were taken from the same crowded stock boxes as the parents of those used in the second trial. The females of *Pediculus humanus* laid 56 per cent. of their eggs on the hair and only 44 per cent. on the flannel, while those of *Pediculus capitis* laid 92 per cent. on the hair and 8 per cent. on the flannel.

In another experiment the attachment of the eggs was carefully studied; as a result it appeared that the method of attachment varied, females of the head louse endeavouring to cement the eggs to a single hair in regular alignment with its direction, while those of the clothes louse attached the greater proportion of their eggs to two or more hairs, for preference choosing those which crossed at an angle.

FECUNDITY.

With entirely unrestricted feeding I am told that an egg production of ten per day is possible for females of *Pediculus humanus*. During my own trials in which the opportunities for feeding averaged about seven hours per day, the greatest number of eggs laid by any one female was 295—an average of 6·4 eggs per day during her adult life. For limited periods this average has often been exceeded, in some instances eleven, twelve or fourteen eggs have been recorded in a single day. It is possible that these figures would be continued day by day under natural conditions. An experiment in which a number of females of *Pediculus humanus* were given the opportunity of feeding for two hours daily in addition to the seven each night, showed unmistakably that additional feeding raised the daily average, while the fertility percentage was not affected.

The fecundity of *Pediculus capitis*, judging by the number of eggs laid in captivity, would seem to be generally of a lower order than that of *Pediculus humanus*. The highest average was only four per day, with a total of 141, but this was easily bettered by females fed twice daily, in later tests, when the stock from which the insects were drawn had been living in captivity for a year, so that we may very reasonably consider that the average is higher under natural conditions, but even the later trials suggest that the ratio is lower for the head than for the clothes louse.

FERTILITY.

Renewed pairing at intervals of not more than twenty days is essential to the fertility of the eggs laid by females of *Pediculus humanus;* this is the ascertained limit. The more usual period would seem to be in the neighbourhood of fifteen to seventeen days. With *Pediculus capitis* the period was generally seven to ten days, at longest, not more than twelve.

A single male of *Pediculus humanus* effectually impregnated eighteen out of twenty-one females, while a male of *Pediculus capitis* fertilized ten—this smaller number was due to the lack of opportunities during his period of greatest vigour, rather than to any want of vitality—the supply of virgin females having temporarily failed.

LENGTH OF LIFE.

Speaking generally, the full adult life of *Pediculus humanus* was about five weeks; that of *Pediculus capitis* under similar circumstances only four.

The longest recorded life of an adult male *Pediculus humanus* was thirty-two days, and that of a female forty-six. The average of a number of females was thirty-four days.

Of the head louse all my records are shorter: one male lived thirty days and a female thirty-eight days, but the average adult life of a number of females was only twenty-seven days.

The length of life of unfed lice depends to a large degree upon temperature and humidity. At a medium temperature such as 60° to to 65° F. the majority of vigorous adult lice live three or four days, a few survived five, and a single specimen seven days. I was informed at one of the infirmaries that their tests had shown a possibility of nine days' survival. As this period agrees with the limit mentioned by both Sikora and Peacock we may conclude that it is correct.

At higher temperatures the survival period without food is shorter; at 76° F. all the lice experimented with died within five days, and at 97° F. within three days. Larvæ just emerged from the egg, unless fed, lived less than twenty-four hours at 93° F., and when kept in a box in the waistcoat pocket the majority lived but little more than a day without food. None survived a second period of twenty-four hours. Cold conditions seemed only less fatal than the hot. When kept at a temperature ranging from 28° F. to 30° F. vigorous adults all survived two days and, though a few showed signs of life after four days, they died without feeding. None of the active lice survived a week in the cold room.

So far as my experience goes nymphs and larvæ, when about to moult, usually show a slightly longer survival period than adults.

Habits.

When feeding, lice are as wasteful as fleas, without the excuse of the latter that fæces consisting of only partially digested blood afford nutriment for their young. In comparison with the bed-bug the digestion of lice appears extremely superficial, and there seems to have been but little development in the direction of the storage of a reserve supply of food, but, as with *Cimex lectularius*, in which this has taken place, provision exists for carrying over the blood in the alimentary canal through the moults, from one stadium to the next. They are sedentary feeders and appear to rely upon the efficiency of the salivary fluid they inject to dilate the capillary system of their host if they fail to penetrate a vein. If a number be placed upon a small area some may obtain satisfaction within thirty seconds of penetrating the skin, others have to wait several minutes—occasionally as long as fifteen—before the blood flows.

Proportion of Sexes.

When a number of lice are kept together in a box and allowed to increase, one does not as a rule notice any disproportion in the relative number of the sexes, but with offspring reared from single pairs the proportion of males and females seems to be almost casual. Any proportion, from equality to broods consisting of entirely males or females, may result; this fact has, I understand, been independently discovered by Dr. Hindle as well as by myself. The following figures will serve to illustrate the phenomenon. The small number of offspring reared in some instances is due to the females dying, probably from old age, within a short time of their segregation. The method pursued was to take paired couples from a stock box.

Proportion of Sexes reared from Pairs of *Pediculus humanus* taken from a Stock Box.

No.	Males		Percentage	Females		Percentage
1	1	=	2	43	=	98
2	44	=	49	46	=	50
3	31	=	65	17	=	35
4	*Nil*	=	—	67	=	100
5	119	=	73	43	=	27
6	39	=	66	20	=	34
7	116	=	68	55	=	32
8	*Nil*	=	—	46	=	100
9	54	=	92	5	=	8
10	*Nil*	=	—	49	=	100
11	10	=	71	4	=	29
12	*Nil*	=	—	39	=	100
13	*Nil*	=	—	48	=	100
14	7	=	9	74	=	91
15	42	=	68	20	=	32
	463	=	45	576	=	55

In a numerous colony such a method of reproduction will of course give the same proportion of sexes as would result if each pair produced an equal proportion of males and females. Presumably this departure from the normal method of sex production serves to minimize the dangers of too close interbreeding.

GREGARIOUSNESS.

Body lice, and possibly to a slighter extent head lice, are gregarious. This is more especially noticeable with the immature forms when approaching a moult. When the insects are kept in captivity this instinct is very apparent, but I have evidence apart from this. During the course of numerous experiments dealing with the destruction of *Pediculus humanus* under captive but otherwise natural conditions, I had several opportunities of observing the behaviour of escapes on my own clothing. It is most remarkable that these free insects tended to collect in close proximity to their captive fellows, in spite of the fact that the latter were imprisoned on pieces of flannel, treated with various insecticides, which from their smell should have exercised a deterrent effect. That the attraction was not always sexual was shown by the fact that immature lice collected in the same way.

PHOTOTROPISM.

Both *Pediculus humanus* and *Pediculus capitis* are negatively heliotropic, crawling away from the source of light, or if diffuse, towards any dark object or shadow in their vicinity. Should the source of light be directly above them, and there are no shadows or dark surfaces near, they either crawl aimlessly, or what is more frequent, if they are numerous, cluster together. Their dislike for light affords a convenient basis for methods of dealing with them under experimental conditions. It also affords a means of making rough trials of the value of deterrent substances or fluids, as their dislike for these can be tested in relation to their desire to avoid light.

CLUSTERING OF EGGS.

Female lice, in captivity, exhibit a sort of homing instinct in regard to the deposition of eggs, returning again and again to a particular spot for which they have a preference. Certain facts tend to suggest that it is the eggs themselves which form the attraction, for I found frequently that when I reversed or otherwise altered the position of the piece of cloth on which they were laid in relation to the box in which the insects were confined, with the object of getting the eggs distributed in order to facilitate counting, that my aims were thwarted by the female continuing to add to the original cluster.

HATCHING.

The hatching of the eggs is conditioned by the temperature, and possibly also by humidity, but of this I have no exact evidence.

Of a number of eggs taken from a stock box, and therefore in various stages of development, none hatched in a room the temperature of which fluctuated between 60° and 65° F., while at 76° F. there was considerable mortality, and the period of incubation of the survivors was erratic, and spread over a longer period than usual. Three hatched within three days, while the emergence of larvæ from thirteen others was distributed over the following nineteen days. Eggs from the same batch hatched readily in my waistcoat pocket with slight, if any, mortality. At 98° F. the period of incubation was very uniform, within five days after removal from the stock box. It is probable that the eggs, ready and almost ready to hatch, and also those very recently laid, were killed by the sudden transfer from the box in my vest pocket (temperature about 85° F.) to hotter and much drier conditions.

One thousand three hundred eggs, kept in an incubator under humid conditions at 87° F., showed the following hatching periods: 3 per cent. hatched on the seventh day; 56 per cent. hatched on the eighth day; 33 per cent. on the ninth day; 8 per cent. on the tenth day; and 0·2 per cent. on the eleventh day.

The temperature of 87° F. is probably not far from the normal. I find that a thermometer placed between my shirt and skin in the region of the chest records 93° F.; between shirt and waistcoat, 86° to 87° F.; in the waistcoat pocket, 85° F.; while in the breast pocket of a buttoned coat it was only 74° F. to 75° F. If the thermometer is placed against my uncovered head it records 82° F., and in the folds of a woman's hair, near but not touching the scalp, the temperature is 81° to 82° F., while if the scalp is touched the mercury rises to 89° F. These readings were made in a room kept at a temperature of 62° F.

The similarity of the hair temperature to pocket temperature suggests that the eggs of *Pediculus capitis* are adjusted for incubation at the same temperature as those of *Pediculus humanus*, and this conclusion is in agreement with my experience when breeding both insects under the same conditions.

RESISTANCE TO COLD.

Exposure for seven days to a temperature varying between 30° and 38° F. does not kill the nits, but none survived a period of eleven days, during the last four of which the thermometer fluctuated between 30° and 33° F. Active lice survived two days at 30° to 38° F., and some partially recovered after four days, but died without feeding. None survived seven days.

RELATIONSHIP OF *PEDICULUS HUMANUS* AND *PEDICULUS CAPITIS* TO EACH OTHER.

Cummings, in his pamphlet on the louse and its relation to disease (Economic Series No. 2, British Museum, Natural History), remarks that the clothes louse differs so little from the head louse that it is necessary to indicate carefully its distinguishing characters, and gives the following points: Size: clothes louse: mean length, males 3·19 mm., females 4·14 mm.; head louse: mean length, males 2·46 mm., females 3·03 mm. Form: In the clothes louse the body immediately behind the head is broader than it is in the same place in the head louse. The lateral angles between the segments of the hind part of the body are appreciably sharper in the head louse than in the clothes louse and the clefts which run in from the sides between the segments are deeper. In the female of the clothes louse the gonopods are narrower towards the tip than in the head louse. The antennæ of the head louse are usually thicker than those of the clothes louse. I have noted that the eggs of the head louse are slightly smaller on the average, and have already remarked on the divergence in the egg-laying instincts. To these points must be added the generally observed fact that the head louse clings to hair and the body louse to clothing.

To sum up these points there is a difference in average size and general form of the insects; a difference in minor points of structure of antennæ and gonopods of the females; a difference in the size of eggs and a variation in regard to the instincts connected with their deposition. Exceptional individuals of either race may produce the characteristics of the other in one or other of these points, but statistically the separation between the insects appears to hold.

HYBRIDIZATION.

In an attempt to obtain further evidence bearing on the question of specific identity, hybridization was resorted to; cross pairings between males of *Pediculus capitis* with females of *Pediculus humanus*, and vice versa, were easily brought about, and carried through without difficulty to the F. 3 generation.

The following points are of interest: There was a high mortality among females of *Pediculus capitis* placed with males of *Pediculus humanus;* unless removed after their first union they were invariably killed. The mortality of the males of *Pediculus capitis* placed with females of *Pediculus humanus* was also higher than when paired with females of the same race. There was a marked disparity in the proportion of sexes in the F. 1 generation of the cross *Pediculus capitis* male and *Pediculus humanus* female. Of four pairings the percentages were :—

No.	Percentage of males	Percentage of females
1	74	26
2	86	14
3	51	49
4	76	32

In the reverse cross, and subsequent generations of this cross, no such obvious disparity occurred. In view of the casual sex production in broods of *Pediculus humanus* already referred to, there would be nothing surprising in the above figures were it not that the disparity is all in one direction—namely, a high percentage of males. The small number of broods, however, does not warrant much stress being placed on the phenomena. In size the hybrid insects of the F. 1 and F. 2 generations are approximately intermediate, but extreme disparity in size was noted in some of the F. 3 broods, very small female and large male specimens being noted, in addition to large females, small males, and others of normal sex proportions. Apparently some of the insects had reverted to the paternal proportions, while others retained the hybrid features in this character.

SUMMARY OF FEATURES IN THE LIFE-HISTORY OF PRACTICAL IMPORTANCE FOR SANITARY PRECAUTIONS.

Eggs take seven or ten days to hatch under normal conditions—i.e., in clothing that is constantly worn; if discarded and allowed to cool for a period each day, the time of hatching may be extended for five weeks.

Active lice can exist without food, and apart from any host, for periods of up to nine days.

Young lice take from ten to fourteen days to attain sexual maturity.

Females, after attaining maturity, require two to four days before they commence to oviposit.

Egg production cannot take place without food, or under cool conditions (below 65° F.).

Eggs laid by unpaired females do not hatch.

Impregnation is not effective for more than twenty days.

As many as ten or twelve eggs per day may be laid by each female.

A total of 300 eggs may be laid by one female.

The female after maturity may live for forty-six days.

Before the close of her life a single female may have 4,160 living offspring.

PREVENTIVE MEASURES.

Pediculosis is a sign of, and depends for its continuance upon, a low standard of life. With a change of garments and the institution of the weekly washing of shirts and underclothing, the number of the parasites is speedily reduced; when sufficient means and leisure obtain among the people to allow of the regular change and cleaning of bedding as well as of clothing, *Pediculus humanus* must die out.

As regards *Pediculus capitis*, where the conditions of life are not so hard that the mother of the family has to spend the time that should be devoted to the care of her children in the winning of their bread, the head louse follows its relative into obscurity. Drink and ill-health may provide sufficient primary or secondary victims to prevent its extinction, but will do little more.

If we had not quite achieved this desirable goal when the war broke out, it seemed within measurable distance in the more wealthy of the European States. With the advent of war, armies were plunged back into that condition of barbarism which renders the washing and changing of garments, let alone bedding, an erratic and occasional proceeding, even when it does not prevent it entirely for weeks or months together.

The solution of the louse problem depends therefore either upon campaigning under a civilized standard of life, or upon the adoption of efficient remedies for the destruction of these insects. The plan adopted by the British authorities partakes of both methods. In the West the stable Front has rendered it possible for the troops in the field to enjoy, in large measure, the civilized custom of the washing and changing of clothing, but in the actual fighting line and in other areas this has not been found practicable, and dependence upon insecticides is essential.

It is to be presumed that the necessary orders are issued for the supply of remedies to the men in these situations, but that owing to some accident or flaw in transport, they are not always received. Whatever the cause may be, however, complaints are so numerous as to make it appear to a civilian that the state of our troops in the fire trenches is what may be termed "lousiness," tempered by the receipt of insecticides from relatives or friends.

It follows as a consequence that, in spite of the baths, wash-houses and laundrying establishments, intermittent irritation tends to be general, owing to the re-infection of the already cleansed, or to the infection of fresh troops from home by men straight from the fighting zones—pediculosis being continual in the firing line because the dug-outs and resting bunks are in constant occupation.

The defect in arrangements would seem to be, on the one hand, in the failure of the authorities to issue a sufficient supply of insecticides (if any) to the troops actually in the trenches, and, on the other, to recognize that, while all the known insecticides for the destruction of lice are defective in one direction or another, their imperfections may be counterbalanced by complementary use. To reduce pediculosis to its lowest point it is necessary to use relatively stable, slow-acting remedies, in addition to one of quick action if of short duration. Those most serviceable in emergencies are impracticable for continuous use, owing to the large quantity which their rapid evaporation renders necessary.

The treatment of discarded clothing should, wherever possible, depend upon the action of dry heat, not because this is more effective than hot fluids or steam, but because it is more economical. Wet or moist clothing requires drying before use, and time will not usually permit of this being left to sunlight and open air. A drying chamber, if once inaugurated, might just as well be maintained above the level of temperature necessary for the destruction of lice and nits as below it, especially as it is practically certain that the same temperature would destroy the active females of the Sarcoptes, which cause scabies. The heat necessary for the destruction of both lice and nits is 52° C. for a period of thirty minutes. Allowing a margin for contingencies, 55° C. for this period will be quite high enough if the garments are spread and hung. Higher temperatures will kill more quickly and give greater penetration, but the question of their economy is doubtful, unless the conditions of speed and space render it imperative.

The operation of ironing the seams of tunics and breeches, if performed with due regard to the heat of the iron and the speed of its passage, may no doubt be as effective as it is convenient, when only the simplest of remedies is possible, but if a hot room is available it seems reasonable to suppose that much economy of labour, as well as greater efficiency, could be obtained by hanging the garments in it. If great speed and economy of fuel were desired, a tubular oven with travelling hangers could be arranged on the principle of a biscuit bakery.

In washing or steaming garments or bedding the same temperature as in dry heat is sufficient; higher temperatures will give quicker action, providing thorough penetration takes place. The nits are actually destroyed if they experience the heat of boiling water for half a minute. The addition of chemicals to the water used for washing or soaking is superfluous if its temperature is high enough, and its quantity in relation to the clothing sufficient, to ensure that the nits in the fabric experience 55° C. for thirty minutes, or a higher temperature for a shorter period. On the other hand, if nits and lice can be killed more conveniently or cheaply by the addition of chemicals, it is wasteful to use heat in addition.

I am rather labouring this point, because it is one of the peculiarities of the destruction of insects that economy of thought and extravagance in practice should be so general. The spirit which dominates the illustrations of Mr. Heath Robinson seems also to exert considerable control over the inventors of insecticides, whose common practice it is to endeavour to raise the general efficiency level of their preparations by complexity in combination. The following, though possibly somewhat flamboyant, is a not unfair illustration of the process: "Kummerfeld's wash is useful, and is prepared as follows: Twenty parts precipitated sulphur are incorporated in a mortar with fifty parts glycerine; two parts of camphor are separately ground with fifty of Eau de Cologne, and twenty of borax and 870 parts of distilled water are added; the whole is mixed together, and three drops of an extract of musk are added; shaking in order to prevent the sulphur from settling down; fifty parts of ether are added to the mixture." This principle pervades a large proportion of the pre-war remedies, and some of the recent ones, and has even crept into the work of scientific importance. For instance, it was remarked by one experimenter that cyllin water when cool was not effective, but became so when heated to 60° C.; yet it had already been pointed out in his own paper that dry heat at 60° C. killed the nits.

INFECTED DUG-OUTS.

If practicable the wood-work of which these are built should be flat-oiled; this might considerably reduce the risks of infection, as creosote oil is very deadly to insects, and its application might considerably reduce the risks of lice resting on the wood-work. Treatment with Colorado vermin killer, a preparation that I was asked by the Army Authorities to test in regard to its efficiency against bugs, would be about equally efficacious.

INSECTICIDES.

Insecticides may act either by contact or vapour; in the first case they obtain entrance at the insect's mouth or spiracles and poison it or obstruct the trachea and suffocate it. In the second case the vapour which destroys the insect must poison it by way of the tracheal system. Popular opinion, especially among the compounders of insecticides, has decided that strongly-smelling substances are effective owing to their smell—hence the number of essential oils which are recommended or incorporated. As contacts, any essential oil appears to be deadly, but their action, apart from contact under the practical conditions of use, is likely, in the great majority of cases, to be disappointing, the presence of smell being but a poor guide to the quantity of vapour necessary to bring about the death of the insects.

It will be my endeavour to show that for practical purposes it is necessary to use almost all remedies as if they had a contact value only, because under the conditions of use the diffusion of vapour at a concentration necessary to kill is limited to so small an area that the advantage of the vapour poison over a contact poison is of little if any moment.

My first experiment in the use of insecticides against lice was carried out with 25 grm. of pure flake naphthalene, placed in a thin cotton tube worn as a belt next my skin. I then suspended between my shirt and skin a number of gauze bags containing active lice. These bags, which were kept from collapsing by a wire gauze frame, were placed, both at back and front, about 5 or 6 in. above and below the belt. One bag slipped down to within 1 in. of the belt, and in this bag alone were the lice affected during a nine hours' trial, a few out of a number being killed. The repetition of this experiment under modified conditions showed a similar result. In order to test the comparative value of a number of different remedies under practical conditions and determine their range of action with some degree of accuracy, I constructed a small piece of apparatus to suspend in front of the body, so that the insects could feed during the progress of the tests. In the central compartment was placed a piece of thick lint 1 in. square impregnated with the fluid or substance it was desired to test; lice were placed in all five chambers. The results showed that even with so continuously volatile and effective a substance as naphthalene the vapour action was very feeble, even within a range of 1 in., and practically ineffective at 2 in. While all the insects in the same compartment as the naphthalene were killed within three hours, only 23 per cent. of those in the adjoining chambers were killed within ten hours, and only one individual out of fifty died in the outer chambers during the same period.

More recent experiments, conducted under another method, while confirming these earlier results, go still further in indicating how narrow is the range of effective diffusion. The experiments in question were planned with a view to obtaining a comparative result between different samples of naphthalene; consequently, it was necessary so to adjust the conditions that a proportion only of the insects were killed during the period of trial. Small pockets of thin cotton, 15 mm. square, were stitched on to slips of flannel about 80 mm. square and in these 0·2 grm. of the naphthalene was placed; over this a larger cover, about 47 mm. square, of fine motor veil gauze was stitched, and beneath this the lice were confined. One of the results could only be explained by the supposition that one of the covers was so arranged that it allowed a slightly further range from the source of vapour than did the other. In one case the distances of the margins of the cover from the edge of the pocket containing the insecticide were 14, 17, 18 and 20 mm. respectively; in the other they were 12, 17, 18 and 18 mm. Although the difference was so slight it seemed the only possible explanation of a difference of 17 per cent. in the average mortality during four separate trials.

In order to test the correctness of this explanation the cover was removed from the slip which was giving the higher mortality and replaced with a slightly larger one. The increase in the distance of the sides of the gauze covers from the margin of the pocket containing the naphthalene was 12, 17, 18 and 18 mm. to 18, 20, 21 and 23 mm., the actual increases being 6, 3, 3 and 5 mm. in range. As a result, in place of a mortality of 42 per cent., none of the lice were killed during the normal four-hour period of the trial, and when the period was increased to eight hours only 7 per cent. died.

It is upon the results obtained in these experiments that I base my case that the effective range of vapours between the skin and undergarments is so limited as to be almost negligible. The deterrent effect of the various insecticides experimented with I have been unable to test on a practical scale under the normal conditions of use. It is possible that the vapours set free may have a wider range in deterrent than lethal effect. I am, however, dubious as to placing very much reliance upon deterrents at all, owing to the fact I have already mentioned when referring to their gregarious habits, that of the lice which escaped and enjoyed free range over my body and clothing, the greater number, in some cases all, were found on the flannel slips which had been impregnated with some insecticide. Attracted thither by the presence of their captive fellows the gregarious instincts proved more powerful than the smell of the insecticide was deterrent.

It was also shown by experiment that quite efficient insecticides when rubbed on the skin are of little, if any, use in preventing the insects feeding. The method of test employed was to rub the skin of the forearm thoroughly with the preparation, re-cover the skin area with the sleeve and, after fifteen minutes' interval, allow a number of hungry lice in a box to feed through the gauze cover of the box. In no case did *all* the lice in the box refuse to feed; generally all, or the greater number, fed greedily. Nor were the lice usually affected. In most cases the few deaths which occurred might have been due to natural causes. A considerable mortality, however, followed when sassafras oil was used to anoint the skin. If in many of these cases the same quantity of the preparation had been smeared on the *shirt* and the insects confined under a gauze cover on the area, though it would probably not have prevented their feeding, it would probably have killed them within a few hours, owing to its spreading over the body of the insect and finding its way into the tracheal system.

The only method of testing the differential deterrent action that I have employed so far consists in utilizing the dislike of lice to light in the following manner: A large sheet of filter paper or other rough, fibrous paper, the surface of which enables them to crawl freely, is placed on a table in front of a window. A band of the insecticide is applied to the paper with its ends turning towards the light; the lice are then put down between the source of light and this line, and one can roughly determine, either from their preference to turn back and face the light or to cross the band, which preparation is the better deterrent. As the method has but an uncertain practical value I have only carried out a few trials, but these show that the best deterrents are not necessarily those with the most powerful odours.

It follows, if what I have stated is correct, that however valuable and interesting from the standpoint of pure science many of the published papers concerning the relative efficiency of various fluids, substances or vapours for the destruction of lice may be, they have but small practical value, owing to a want of appreciation of the essential facts connected with their use. Remedies have to be applied between the clothing and surface of a more or less rotund body, the temperature condition there being in the neighbourhood of 87° to 92° F. What effect their vapour may have in closed tins, under bell jars or when the lice are suspended within a short distance above the insecticides is beside the question. All depends upon the concentration and duration of the vapour resulting from the quantity you can afford to apply to any given area and the effective diffusion range around this area.

Having now put before you the basis of my contention that all insecticides used under the conditions named must be considered from the point of view of practical use as contact remedies only, I shall proceed to consider them from this point of view.

The main points for consideration are: first, cost, because it is useless to trouble about remedies which, if used in sufficient quantities to be efficient will cost more in a week or two than a new shirt—a point that has been largely lost sight of by the purveyors of remedies under a trade name. Secondly, speed of action in relation to its duration. Generally speaking, speed of action and period of duration are in inverse ratio to each other, but the relation is not always a simple one, and may be modified. Very volatile substances and fluids are of little use unless they can be applied to the whole area of the underclothing at one time, which is seldom practicable while in wear, because they evaporate so rapidly that the treated area is free for re-colonization within a few minutes. On the other hand some substances are so slow of action that, although they might very considerably reduce the length of life and breeding capacity of the insects, the user would be continually subjected to irritation by the sick, and by new recruits which had strayed on to him from outside sources.

There is a further point to be considered that is frequently overlooked by the makers of preparations, both professional and amateur, and that is, how the preparation is to be used. Persons devoid of knowledge of the bionomics of the louse, as well as those who have inadequately considered the subject, appear to think that any preparation, whether fluid, paste or powder, can be rapidly applied by dusting or anointing the skin surface with it, probably the most unpleasant and wasteful method that can be adopted. For, in the first place, one gets all the discomfort and danger of skin irritation, and in the second, if the preparations are at all volatile, they are even more quickly dissipated than would be the case if used on the under-garments.

The question of the best method of use is not always easy to answer, and the difficulty with some very efficient remedies, especially quickly acting, but rapidly dissipated oils, is to devise some means of distributing them evenly over the garments in quantities sufficiently small not to be disagreeable or wasteful, if possible using some vehicle for this purpose which, while retarding their over speedy action, renders it effective over a longer period. For this purpose soap, if it can be used, is very useful as it supplies an easy means of making a paste, while if an emulsion of the remedy with soap is possible, it makes impregnation by watery solution of any strength possible. With other contact remedies again soap enhances their action as it makes contact with and spreading over the wax-like surface of the insects' integument more easy and certain. There is also the final advantage that it will aid in removing the dirt, which accumulates on a greasy surface, when the garment is washed.

For the efficient protection against the breeding of lice in garments, the underclothing should be impregnated with some fluid or substance which will retain its effectiveness for upwards of a week, and as a precaution against enforced delay in re-treatment, an effective and easily applied remedy of quick action should be carried in addition. I am assuming that the thorough impregnation of undergarments can only be carried out in a large camp or base behind the fighting line. Of all the remedies with which I have experimented, the only ones which fulfil the first of these conditions experimentally and are sufficiently low in cost are crude carbolic acid, consisting chiefly of cresols with a trace of phenol and tarry oils, and cresol—the latter being slightly less efficient. The most feasible and effective method of use is to emulsify them with soft soap, using from 45 to 50 per cent. of soap to 50 to 55 per cent. of the crude carbolic. A perfect emulsion is produced by using sufficient heat thoroughly to melt the soap. The correct strength of the solution to be used for impregnating the garments ranges from 5 to 10 per cent. according to the climate or period of the year. If less than 2½ per cent. of the crude carbolic or cresol is used the destruction of the lice is slow and faulty, if more than 5 per cent. irritation may be caused to the skin in warm weather. The action is by contact and the soap renders this the more speedy and certain. Under cool, dry conditions effective action is slower or ceases entirely because the perspiration is insufficient to moisten the garment and set free the emulsion to act on the insect. As the lice tend to congregate to the less ventilated and more humid situations the impregnation tends to be most efficient where it is most needed. During the summer I found that a shirt treated with a 5 per cent. solution—2½ per cent. crude carbolic—was efficient in the destruction of lice for a period of five or six days—the action being quicker or slower from a few hours to a day, according to the amount of perspiration. During the winter a 10 per cent. solution—5 per cent. of crude carbolic—was necessary to produce the same results. It would follow that the less the sweating the longer the period of action. Treated flannel may be kept at least fifteen days and probably much longer before use without losing its efficiency. An average sized shirt has an area of about 1,600 square inches, and will take up about 1,000 c.c. of fluid, while after wringing it will retain about 500 to 600 c.c. Thorough drying of the garment is essential before wearing. The individual treatment of garments by men is not very practical, and if many garments have to be wrung, either rubber gloves or a mechanical wringer is necessary. The cost per shirt for solution at pre-war prices would amount to about ½d. to 1d. according to the strength of the solution used. The fault of this remedy is that it is rather slow, and does not prevent feeding until in full action.

The most efficient quick-acting remedy of low cost I have used is naphthalene, which for the purpose of the destruction of lice is quite as effective, and for some methods more effective, in its crude cheap form than in its more expensive purified state. It acts by vapour and possibly also when mixed with oil, soap or grease, as a contact. But it is no exception to the general rule of very restricted range of effective action, it must be *thought of* for practical use as if it killed by contact. Under the conditions of use between clothing and body its action is very rapid, killing in about two or three hours if used in sufficient quantities, and feeding seldom occurred during tests. Unless continued action is desired the quantity used need not be large, as its efficiency depends upon the surface exposed to evaporation; it is consequently more rapid in action when used as a powder, or if the fabric is impregnated with it, than if put up in packets.

Naphthalene is, however, so efficient that the retarding of its evaporation may be a decided gain under circumstances where continued local action is desired. The impregnation of garments or blankets with it is not difficult, as it is readily soluble. Benzine will take up about 40 per cent.; methylated spirit between 5 and 10 per cent.; paraffin up to 15 per cent. But, more important still, it can be dissolved by heat in oil or oily fluids, which can be subsequently emulsified and used to impregnate clothing, &c., in watery solutions while warm.

If used in impregnation it is less wasteful than if used as a powder, because in the latter case it dusts out, but under either system the duration of its effect is very short owing to its rapid evaporation at body temperature. At a concentration of 10 mgrm. per square inch, equal to about 16 grm. per shirt, it will within two to three hours kill all the lice present, but will have entirely lost its lethal action within five hours and its smell within eight. On the other hand, if used in packet form, although its evaporation period is prolonged, its range of action is limited to a radius of about an inch from the packet. It is therefore unsuited for the purposes for which the crude carbolic soft soap emulsion method is so well adapted, but as an adjunct to these for individual use in case of emergency it is invaluable, and might be supplied both as a powder for use by dusting a pinch or two in at the neck, or put up as a paste by mixing with soft soap for the purpose of smearing along the seams of garments. For the latter purpose crude naphthalene is even more serviceable in its "unwhizzed" than in its "whizzed" condition, because the quantity of oily fluid which it contains renders the use of less soap necessary to produce a paste. The soap, whilst restraining the speed of evaporation of both the oily fluid and the naphthalene, does not prevent the preparation spreading in the fabric of the garment, under which circumstances it acts as though it had a contact effect. It is possible that this is really the case, but its increased efficiency in range of action may be due to the enlarged area from which vapour is produced.

Having now put before you the facts, so far as I conceive them, concerning the most efficient methods of checking pediculosis in the Army, at a reasonable cost, I propose to deal shortly with some of the other remedies that have been recommended for use against these pests.

CLAYTON GAS.

There is no doubt as to the efficiency of this means of killing active lice or mites in discarded clothing or bedding, but I am still doubtful, in the absence of detailed reports, of the exact procedure and control of experiments, as to the destruction of nits by its means. Nits are very susceptible, and easily killed at certain stages of their development, and, relatively, very insusceptible at others, therefore the possibility of the nits being sometimes killed by the treatment and at others not, is considerable. The chief objection to its use, however, is, I conceive, that of cost and special apparatus to ensure sufficient concentration, while, I suspect, though I confess to having no actual experience, that the time required is much longer than would be needed for dry heat to produce the same result.

Most of the other suggested methods for the treatment of discarded clothing would seem to require even greater elaboration of method and expenditure of money.

INSECTICIDES FOR USE WHILE THE INFECTED CLOTHING IS IN WEAR.

Iodoform and Cytisine.—The former, if it could be used in sufficient quantity, might be ideal, but the cost of impregnating garments with it puts it out of the question, whilst in small quantities it only creates smell and causes expense without increasing the efficacy of other remedies. Cytisine is not only too expensive, but also might possibly prove dangerous.

Vermijelli.—The quantity and method of application required for efficient protection would render this remedy not only expensive, but disagreeable. It is questionable whether it acts otherwise than would any heavy grease, such as the rancid butter of the Cossacks. It is certainly ineffective if used up to 10 per cent. strength to impregnate clothing.

Sulphur.—I am by no means so sure that this is so devoid of action as some experimenters have suggested. Under the condition of use on the body it seems to have a very slow action; variable results are possibly due to differences in the humidity of the body. It is, however, hardly worth troubling about while reasonably cheap and definitely effective remedies are to hand.

Anthracene.—This was tried on account of its similarity of molecular structure to naphthalene, but was all but useless.

Tar Oil.—This is very effective if used neat to impregnate flannel, but is not very suitable for this purpose. If it could be evenly sprayed on in small quantities it might afford a practical remedy of some value. Its period of effectiveness when flannel has been thoroughly impregnated with it is about fifty hours while in contact with the body, but when used in smaller quantities the period would be proportionally less. It can be emulsified with soft soap in the proportion of twenty-five parts oil of tar and ten parts of soft soap, but is not effective when used as a watery solution to impregnate garments, even at a strength of 50 per cent. Presumably, therefore, it acts by its mechanical effect only.

Sassafras Oil.—This is very deadly as a contact and, judging by smell, is one of the ingredients of many of the effective proprietary pastes and fluids. It is one of the few remedies that will kill lice when they are fed on a patch of skin treated fifteen minutes previously. The proprietary pastes and fluids with which I have experimented, however, do not seem to contain it in sufficient proportions to be effective when subjected to the skin test. Economy in its use may be obtained by diluting it with methylated spirit or paraffin (lamp-oil). It can also be emulsified with soft soap as follows: One part soft soap is melted in five parts of water by heat; twenty parts of sassafras oil are then added very gradually, with thorough shaking or stirring. This emulsion can be diluted with water, but does not remain in emulsified condition for more than a few hours after dilution. Flannel impregnated with a 10 per cent. solution, and then dried, killed all the lice within a few hours during the first thirty hours of wear, but after forty-eight hours it was only partially effective during a twenty-four hours' trial.

Legroux's Method.—A trial was made with the impregnated sachets as supplied for use in France. The ingredients are oils of lemongrass, pennyroyal and eucalyptus, to which powdered naphthalene is added. The result was partial and uncertain, the average mortality being only 20 per cent. in an eight hours' trial, while the fact that there were no feeble, but only dead and active lice, raised a suspicion that the small number of deaths which occurred were due to misadventure—probably pressure.

Only a few of the proprietary insecticides have been tested; of these the best known is probably "*Parasitox*," 1s. per stick weighing 43·50 grm. The proprietors' direction is to "rub daily on the inside seams of the clothing and on the body, especially the hairy parts," I have only tested in so far as regards the first half of the direction, the latter portion being, in my opinion, impracticable for reasons already set forth. Slips of flannel were smeared with the preparation and lice imprisoned under gauze on the treated area. The slips were then pinned to the inside of the shirt, so that the insects could feed through the gauze during the period of the test. Used to the extent of 23 mgrm. per square inch, all were stupefied in one and a half hours;

at the close of the twenty hour trial all were dead. A second test on the same piece of flannel showed all but one out of twenty-five lice active after three hours, mostly active after eleven hours, and after twenty-two hours twenty-two living, mostly active, two paralysed, one dead. Used at 10 mgrm. per square inch, a few were stupefied after one and a half hours. After five and a half hours all were stupefied. After twenty-two hours fifteen were living and active, ten were dead. The quantity used would therefore need to be between 10 and 20 mgrm. per square inch, and as a shirt has an area of some 1,600 square inches it would, I judge, be an expensive remedy to use daily.

An insect powder named "Muhak," cost not stated, was dusted on to flannel to the extent of 20 mgrm. per square inch, and the lice imprisoned as before. It stupefied a number in one and a half hours. After twenty hours, eight were still living, seventeen dead. A second trial with the same piece of flannel showed all active after two and a half hours, and also after eleven hours. After twenty-four hours, twenty-two were living, sixteen of which were active; two were dead. Feeding took place during both trials.

A powder supplied by Messrs. Shotter and Jones was tested by the same method. After one and a half hours all were living, mostly active; after seven hours all living, mostly active. After twenty-two hours, twenty-three were living and active, two were dead.

"Meville's" Fluid.—Used as recommended for anointing the skin. A number of lice were fed on the skin area that had been treated with the neat fluid fifteen minutes prior to the test. The insects fed and showed no ill effects. Used neat to impregnate flannel, the trials being subsequently carried out with lice imprisoned under gauze on the flannel, which was pinned to the inner side of the shirt so that the insects could feed, the period of efficiency, as a slow-acting insecticide, continued for seven days. First day after impregnation, 100 per cent. killed within a few hours; second day after impregnation, 100 per cent. killed after eighteen hours; third day after impregnation, a number were killed within forty-eight hours; fifth day after impregnation, ten out of eleven were killed within forty-eight hours; seventh day after impregnation, seven out of thirteen were killed within forty-eight hours. It is probable, however, that its cost would prohibit its use for the impregnation of garments.

Two pastes and a fluid put up by W. A. Proctor, and intended for use by application to the skin, or smearing on garments, were tested. A patch of skin was smeared or wetted with the preparation, and after fifteen minutes hungry lice in a gauze covered box were allowed to feed on the prepared skin area. The pastes had little, if any, deterrent effect; the insects fed heartily and were not harmed. The fluid had a partially deterrent effect, but some of the lice fed heartily—one out of fifteen died within twenty hours.

Used for the treatment of flannel, all the preparations proved to be active insecticides if applied in sufficient quantities, but their effectiveness soon deteriorated when used between clothing and body, so that after twenty-four hours they were only partially effective in a twenty hours' test.

"*No Germo.*"—0·260 grm. was placed in a small pocket on a slip of flannel and the lice confined under a gauze cover above it; the pocket holding the preparation was 15 mm. square, the gauze cover above had an area of 2,246 mm. Under these circumstances crude naphthalene would have killed from 70 to 80 per cent. of the insects within four hours. The record of "No Germo" is as follows: After a four and a half hours' trial no effect; after a twelve and a half hours' trial, four out of thirty were dead.

Two trials were made with the "*Kergold anti-vermin body belt*," for which the suppliers make the following claims: "Perfect immunity from all insect pests. The Kergold is the only belt that affords instant and permanent relief from the bane of the soldier on active service. Vermin simply cannot exist when the Kergold anti-vermin body belt is worn; protects the wearer from head to foot. Total immunity from all further attacks. The medical properties of the belt last for approximately six months." The first trial gave results so greatly at variance with these claims that a second belt was purchased in order to avoid the risk of the first one being a "bad egg." In the first series of trials lice were confined in gauze pockets fastened to the shirt so that the insects could feed during the course of the test. In all, eight pockets were placed at varying distances above and below the belt, from the neck to the thighs; during the course of a continuous twenty-four hours' trial the only lice which died were a few in one of the pockets on the upper part of the chest, apparently from pressure of the braces.

In the second trial much trouble was taken to wear the belt before trial and to induce a perspiration by work in a hot room at 97° F. Four gauze pockets containing lice were suspended within an inch of the belt and most of the insects fed heartily at intervals during a twenty-four hours' test. After this period it was found that out of sixty lice nine were dead, four were feeble, while forty-seven were active. While conceding the possibility that the dead and feeble were due to the action of the belt, I incline to the view that they were overlaid during sleep.

After the belt had been continuously worn for sixty-two hours another test was carried out. After twenty-four hours there were three dead, two feeble and twenty-nine active lice in the pockets. The belt was then worn for a further period, and after five days' continuous wear it was given a last chance. After a twenty-four hours' trial, during which many of the insects fed heartily, one was dead and twenty-nine were active, including one pair in copula.

PREVENTIVE MEASURES AGAINST LICE.

Heat.—Dry air or water at a temperature of 52° C. will destroy both active lice and nits within a period of thirty minutes. Higher temperatures kill more quickly; water at 100° C. kills the nits in half a minute. In the destruction of lice by heat the all important factor is penetration. Thick or folded garments require longer time than hung or spread ones. Bundled clothing is only slowly penetrated. Other factors being equal, dry heat is more economical than wet, because wet garments require drying after treatment.

Cold.—Exposure to cold, 30° to 38° F. or $-1{\cdot}1°$ to $3{\cdot}3°$ C. for two days is fatal to active lice, although they may be living at the end of the period. If continued for four days they are killed within the period. Nits survive exposure to the same temperature for seven days, but not eleven.

Insecticides: Contact.—To keep continuously worn garments free from lice the best method is to impregnate with cresol or crude carbolic acid, employing in soap emulsified form, dipping the garments in a solution containing from 2½ to 5 per cent. according to the climate or season; hot weather needs less, cold more.

Vapour.—Insecticides which act by vapour have a very restricted range—not above 1 in., or, at the outside, 2 in. Naphthalene is probably the most economical and effective of the quick remedies, but at body temperature it rapidly evaporates. If underclothing is impregnated to the extent of 2 mgrm. per square centimetre—10 mgrm. per square inch, or about 16 grm. per shirt—its lethal effect will have entirely passed away within five hours. If used in packets its range of action will be less than 2 in. Used as powder it is wasteful, owing to its dusting out of clothing; mixed with soft soap or grease this waste is prevented and its efficiency is increased. Crude naphthalene is cheaper and more effective than pure. Sassafras and other essential oils are very effective, but kill by contact, not vapour, under the conditions of use. Smell is no guide to vapour effect.

THE DESTRUCTION OF NITS.

Paraffin Oil.—Immersion for five minutes kills the greater proportion, but not all.

Sassafras Oil.—Immersion for five minutes kills all. A 10 per cent. watery solution of this oil in emulsified form with soft soap is also effective as against nits. (One part soft soap melted in five parts of water by heat, twenty parts of the oil added very gradually with thorough shaking or stirring.)

Crude Carbolic Soft Soap Emulsion.—Immersion in a 5 per cent. solution for five minutes kills all.

Tar Oil Soft Soap Emulsion.—Immersion in a 5 per cent. solution for five minutes kills all.

Potato-paring Infusion.—Recommended by a writer in the *American Journal of Clinical Medicine.* Immersion for thirty minutes was quite ineffective, and did not even kill active lice.

Quassia Chips.—An infusion of 20 grm. in 250 c.c. of water boiled for over an hour was tried; immersion for ten minutes was quite ineffective, and the active lice lived for two days afterwards, but failed to feed.

Chlorine Gas.—Nits exposed to the action of this gas during the course of a trial at the Royal Army Medical College hatched in the normal course and adults were reared from them.

Colorado Vermin Killer.—Carbon disulphide, 10 c.c.; crude carbolic, 91 c.c.; oil of tar, 2·5 c.c.; kerosene, 435 c.c.; killed all nits immersed for five minutes.

Of these remedies tar oil emulsion and a 5 per cent. solution of crude carbolic soft soap emulsion might be used for the treatment of heads, as they are more effective than paraffin oil and cheaper than sassafras oil.

For the treatment of nits in the seams of clothing either of these or the Colorado vermin killer would be effective; the latter would, of course, be too irritating for use where it comes into direct contact with the skin.

DISCUSSION.

Professor G. H. F. NUTTALL, F.R.S.: Having been engaged for a considerable time in studying the louse problem I can perhaps appreciate the value of Mr. Bacot's work better than most of his hearers. Apart from my own investigations I have taken the pains to collect the literature of the subject and ere long hope to publish a full account of what is known of the louse and its misdeeds. So far I have notes on upwards of 520 papers and publications dealing with lice. The literature is remarkable, in that most authors completely ignore the work of others that have preceded them. I may say at once, however, that Mr. Bacot's work is in some ways the best that has been done hitherto on the biology of *Pediculus*. I would mention here that three papers dealing with the biology of the louse will appear next week in *Parasitology*[1] from the pens of Mr. Bacot, my assistant Dr. Hindle, and myself respectively. I would refer those interested in the subject to these papers, since they contain much that is new:—

(1) Are *Pediculus humanus* and *Pediculus capitis* separate species? Mr. Bacot has given us biological differences of his own finding besides those usually quoted. He cites Cummings as an authority for certain morphological differences. All of the differences noted by Cummings, excepting that affecting the form of the gonopods, have been given by other observers in the past. I would note that Meinert and Neumann, two very careful observers, failed to find any constant differences. My opportunities of examining material have been limited, but so far I have found no constant differences between the two purported species collected in four continents. I shall be grateful to persons in different parts of the world who will supply me with a large number of specimens, well preserved in 60 to 70 per cent. spirit, and accompanied by full particulars as to their source. From what I have seen I incline to the opinions of Meinert and Neumann that head lice and body lice are but races of one species.

(2) The influence of temperature on the rate of development is a subject of practical interest. Rubner, who made careful studies on the temperature beneath human clothing, determined that about 32° C. represents the temperature at or near the skin surface of a clothed individual when he is feeling comfortable—i.e., neither chilly nor warm. Mr. Bacot, no doubt with cruder methods, has determined 31° C. to be the temperature in question. From my observations, and those of others, I take it that the optimum temperature for the development of lice is about 32° C. I have found that the eggs kept at 32° C. continuously near the skin, hatched mostly after seven days; at 30° C. in the thermostat, hatched mostly after ten to twelve days; at 37° C. by day and 14° C. by night, hatched after fifteen days; at 30° C. by day and 10° C. by

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[1] *Parasitology*, February, 1917, ix, pp. 228, 259, 293.

night, hatched after twenty-seven days; at 30° C. for twenty-four hours, and at 5° to 10° C. for twenty-four hours, alternately, hatched after 35 days.

(3) I have failed to observe any difference in the manner of oviposition of "*capitis*" and "*vestimenti*" upon hairs.

(4) Mr. Bacot's statement that additional feeding of adults leads to a greater number of eggs being laid is confirmed by my observations. When fed twice daily I found, as most observers have, that a female lays four to five eggs per day. Mr. Bacot, when he fed them more frequently obtained an average of 6·4 eggs per day; at times this number was much exceeded. I have, however, obtained better results under the optimum conditions offered by keeping lice confined continuously in a felt cell strapped to the arm. Under these conditions of unlimited opportunities of feeding, and an optimum of warmth and moisture, two females laid an average of ten eggs apiece a day for twenty successive days; one female laid 266 eggs in all before she died, probably from being crushed; nevertheless her ovaries were not exhausted.

(5) *Longevity.*—I have also found that adults may live four to five weeks when fed twice a day. The longest period recorded for lice surviving unfed is ten days, this observation having been made by Hase. Observations as to alteration in the fat-body of lice that are starved are lacking.

(6) The remarkable difference in the proportion of the sexes in various broods, to which Mr. Bacot has referred, was made the subject of special study by Dr. Hindle, and will be found described with full protocols in his paper.

I would note with regard to the duration of the life cycle of lice under normal conditions upon man, that my experiments have shown the development from egg to egg to last seventeen days. In the experiment made by Warburton (1911), who carried lice in receptacles near his person, feeding the insects twice daily, the corresponding period was twenty-four days.

Turning to the matter of dealing with the destruction of lice, I would note that my experiments and a survey of the literature published in all the countries engaged in this war, have both led to the conclusion that *dry heat*, where applicable, is the quickest, cheapest, and safest way of dealing with infested articles of clothing, furs, bedding and the like. I find that moderate dry heat kills all lice and their nits in a short time; they are certainly killed in one minute at 65° C., or in five minutes at 62° C. Immersed in water heated to 76° C., they are killed in half a minute, at 60° C. they are killed in five minutes. In practice, naturally, time must be given for heat to penetrate the objects which are treated. The time required will depend upon the degree to which the objects are packed together, as old experience at the hands of bacteriologists has shown. The *hot air should circulate* freely among the objects to be treated if rapid destruction is to be attained. Various simple ways of dealing with this mode of louse destruction are in use to-day. I shall deal with the subject on a future occasion. The *storage* of infested clothing and blankets in a dry warm room would rapidly lead to the death of vermin. I heartily endorse Mr. Bacot's remarks upon the useless character of most insecticides and repellants. I would add to his list an article advertised as the

"Asiatic body cord," which is said to work wonders: it consists of a string to be tied around the waist, the string being surrounded by a greasy substance having the colour of blue ointment; smeared on a slide and examined microscopically, I detected globules of mercury in it. Naphthalene, as also sulphur, have not hitherto given complete satisfaction, and no little trouble abroad. All the evidence points against the use of sulphur dioxide because of its injurious effects on fabrics and metal, &c., and the smell that clings to articles treated with it. Carbon bisulphide vapour has been found better than SO_2; its odour vanishes rapidly, but is objected to because of its toxicity and inflammability. My experiments carried out last year with twenty-four hour old nits and a number of insecticides will be published in due course, but I may mention a few of the results: Nits were not killed by immersion for twenty minutes in paraffin; ten minutes in benzine, petrol, ether; five minutes in carbon bisulphide. Nits were killed by immersion for ten minutes in 2½ per cent. carbolic acid or in carbon bisulphide; by five minutes in 2 per cent. lysol; by one to two minutes in sublimate vinegar or sublimate glycerine. The use of 5 per cent. carbolic as a head wash, applied for ten minutes, was advocated by Whitfield (1912) for the destruction of *Pediculus capitis*. Cresol soap, to which Mr. Bagot refers, has been used with apparently satisfactory results in the German army, and its use was advised in this country by Copeman. It will be interesting to learn how the method suggested by Mr. Bacot of applying carbolic acid to underwear will work out in practice. Experience in the field has shown that different methods of dealing with the problem are required, and that they must adapt themselves to the most varied conditions. At times the conditions are such that the merest palliatives are all that can be used. Even hand-picking, if persisted in, has given good results. Finally, a word about dead or empty nits adhering to cloth; I have been asked how these are best removed. They can only be removed mechanically by means of a knife blade or finger nail, or by singeing off the fibres to which the nits adhere; no solvent affects the chitin-like cement which glues the egg to the fibre without first destroying the fibre.

Professor LEFROY: I am astonished that Mr. Bacot makes reference to only one published work, and that now, at this stage of the War, he can discuss this problem, and can bring forward no newer remedy than naphthalene, and does not even give the credit due to Major Lelean, who originated the N.C.I. formula in the winter of 1914-15 or earlier. In January, 1915, it became known to us at the Royal College that vermin were really bad at the Front; that the material used in India as crude oil emulsion was fatal to lice, and had been successfully used there for some years, both against head lice and body lice. It was also essential that a special grade of mineral oil, containing a definite hydrocarbon, should be used, as neither petrol nor paraffin were deadly to lice as vapours. I had some emulsion made up, and on my recommendation the Army Medical Service agreed to make a trial of it. This oil emulsion came into use under the name of "Vermijelli," and was tested in France by Lieutenant Gair, with some fifteen or twenty other preparations. He reported in favour of N.C.I. and oil emulsion jointly, and I am informed that these were officially issued at the rate of ⅔ oz. per man per week. While in India I had this preparation made at the Medical Stores Depot, Bombay, and issued for use in Mesopotamia, and I still adhere to the opinion that this emulsion destroys lice if used on the body; kills eggs if applied to them; keeps lice from infecting you if the underclothing is impregnated with it. I have never claimed that clothing impregnated with it will kill lice. I know of nothing that can make clothing deadly to lice except certain oils, the application of which presents difficulties. Mr. Bacot, when referring to vermijelli, says: "The quantity and method of application required for efficient protection with this remedy would make it not only expensive but disagreeable. It is questionable if it acts otherwise than any heavy grease, such as the rancid butter of the Cossacks would do. It is certainly ineffective if used up to 10 per cent. strength to impregnate clothing."

Mr. Bacot's sole test of efficiency apparently is that the preparation must be applicable to clothing, and that this clothing will then, if worn, clear you of lice. This is not, in my opinion, the louse problem.

Mr. Bacot bases his remarks on tests made with lice enclosed in muslin bags placed under his clothing; but he does not seem to have made the proper test, which is to become verminous and then treat oneself. His laboratory conclusions, based on a defective method, are contrary to the experience of men at the Front in hundreds and thousands—men, officers, and ladies, from France, Serbia, Mesopotamia have testified in scores to the efficacy of the oil emulsion, and I still maintain that an emulsion of fuel oil of the specific gravity and boiling point that I refer to is an efficient destroyer of lice, if applied on the body in sufficient quantity and vaporized under the clothing with the heat of the body. If otherwise, why has vermijelli proved effectual? Why did the Army Medical Authorities adopt it? For it has filled a gap at a critical time in Serbia, France, and elsewhere. There are doubtless better specifics now, but it is not fair to dispose of the oil emulsion as Mr. Bacot does. The solution of the problem is not made clear by the lecturer; the last paragraphs of his paper refer to many methods, and apparently leave the choice open. What treatment does Mr. Bacot recommend to men who are verminous and to those exposed to vermin infection? If he were sent to a Front what would he do? In such circumstances I provided myself with the oil emulsion discarded by Mr. Bacot, and I found that it worked. The method of keeping lice in gauze pockets fixed under the clothing does not appear to be any real test; the louse is very sensitive, and there is only one real test: *be verminous and clear yourself.* But Mr. Bacot relies on an experimental method which is of doubtful value. The advice given by Mr. Bacot appears to be that the soldier at the Front should rely on the doubtful remedies sent to him by his relatives. But as this paper is entitled "The Louse Problem," it is reasonable to ask what is to replace the methods which have proved so far reliable.

I would further suggest that there is scope for further work, that the limits of the investigation are perfectly definite, and that the body temperature is the one important factor. The Germans are reported to clear their men with cyclo-hexanone ($C_6H_{10}O$), a compound related to what I believe to be the active principle in the fuel oil used. If I were to attempt further work with the louse, I should commence to investigate the cyclo- and allied compounds contained in these oils. Mineral oils are composed of a great range of hydrocarbons, and their insecticidal action varies enormously; the fractions that come off at temperatures between 250° and 400° from the different oils differ in chemical composition, and it is amongst these that I believe the best odourless liquid will be found that will kill vermin at body temperature. I thought we had it, but Mr. Bacot does not agree. It is perhaps going far to dive into the difficult chemistry of the oils in order to obtain a vermin remedy. Perhaps I am mistaken, but Mr. Bacot appears to lump all these together; if so, I understand his lumping together fuel oil and the Cossacks' rancid butter.

Lastly, I hope the Section will not confine its discussion to the academic and scientific aspect of the problem. The thought that there are hundreds and thousands of men scratching themselves at the Front fills my mind to the exclusion of the purely scientific aspect of this work, and I look to the attainment of some practical conclusion that will help them. I trust that this paper and the discussion may not end in talk only but in some real advance in methods of treatment.

Mr. A. W. BACOT (in reply): Of special interest and value are Professor Nuttall's observations relative to the minimum within which the life cycle may be completed; his figure of seventeen days is some three days less than the minimum of my own series, and corresponds to a decided acceleration of the rate of increase in offspring resulting from a single pair.

Professor Lefroy's observations raise points which the practical importance of the problem compel me to examine in some detail. I can, however, pass over briefly the accusations of ignoring the work done by previous investigators. So far as these relate to the purely biological aspects of the louse problem it is sufficient to remark that this paper only treats of them incidentally, and that in the special memoir on the scientific questions involved, which I have contributed to the current number of *Parasitology*,[1] the necessary references will be found. As to the priority of Major Lelean in connexion with the practical use of naphthalene, a professor of entomology must surely know that naphthalene had been employed as an insecticide by hundreds of persons years before Major Lelean dealt with the subject, and that to complain of the omission of any individual's name in connexion with it is as reasonable as to charge a writer recommending opium, without cited authorities, with doing an injustice to Sydenham. The gravamen of Professor Lefroy's charge is, however, threefold—viz., (*a*) That my experimental methods are faulty; (*b*) that I have brought forward no newer remedies than naphthalene; and (*c*) that I have been unjust to the claims of the proprietary article "vermijelli."

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[1] *Parasitology*, February, 1917, ix, p. 228.

(*a*) I need not recapitulate the experimental technique carefully explained in the text of my paper. It is certainly true that a remedy found effective as against lice confined in the manner described might not be effective against the whole of a louse population allowed to wander fancy free over one's body. But it is not less true that a remedy which fails under this method of examination will be futile when the lice are given a still better chance of escaping its influence. My technique clearly provides the minimum standard to be reached. If Professor Lefroy has evolved a better technique, it is his duty to publish it so that other investigators may be able to give it a trial. Vague references to the experience of even "thousands of men, officers and ladies from France," or elsewhere, carry no more weight than the similar utterances to be found in the advertisement columns of the daily press.

(*b*) The charge that I have brought forward no newer combination than naphthalene as a subject for trial should be weighed against the fact that the paper deals with a long series of tests performed with various substances, and a detailed method of impregnating underclothing with a solution of an emulsion of crude carbolic acid and soft soap. I have, of course, no personal interest in the newness or otherwise of any remedy. Professor Lefroy indeed says, in reference to "vermijelli," that "there are, doubtless, better specifics now," but he has failed to mention any, and there is no published evidence that he has performed a single test with any substance other than crude mineral oil emulsion; indeed, I cannot find that even with respect to this heavy oil emulsion, Professor Lefroy has made public any controlled experiments similar to those regarded as essential in ordinary biological or pharmacological investigations. One would perhaps have thought that such elementary desiderata as range and period of action, effect of physical conditions, effective concentration or quantity in relation to area, would have been made public by an investigator so solicitous of bringing comfort to the "thousands of men, &c.," and I must regard the other "thousands of men, officers and ladies, &c.," as but phantom substitutes for this exact information. It is perhaps possible to be scientific without being in Professor Lefroy's sense of the word academic.

(*c*) Professor Lefroy's eloquence in no way moves me to weaken what he evidently regards as a censure of "vermijelli." I pass over his suggestion, rather than explicit statement, that "vermijelli" derives specific virtues from containing certain hydrocarbons of specific constitution; no one, not even the "thousands of men, officers and ladies," having produced any definite evidence on the point. He opposes to my remark that "vermijelli" is certainly ineffective, &c., the statement that "I have never claimed that clothing impregnated with it will kill lice." By way of commentary on this positive assertion I will quote a passage from a pamphlet written by Professor Lefroy and widely circulated some months ago.[1]

> "The emulsion should be used in the hospital laundry as soap, or in combination with other soaps. The articles, after washing, should be rinsed in water containing from 1 per cent. to 2 per cent. (about 2 oz. to the gallon) of the emulsion, well mixed. They should not be wrung out too tightly, but allowed to dry so that the clothing is impregnated with it. The clothes will then confer protection against lice. Vermin hatching out from the eggs upon cloth that has previously been treated in this manner have been found *incapable of survival.*" [My italics.]

The difference between claiming that a substance will kill lice and claiming that it will render them incapable of survival is so subtle that I think it a little hard to be condemned as unpractical and academic by a writer who lays stress upon it.

To sum up, I think the claims made on behalf of "vermijelli" by its vendors are in excess of its real merits. My reasons for this opinion are set out in the paper just read, and subsequent experiments simply confirm these results. When Professor Lefroy sees fit to make a quantitative series of trials and publish the results I shall willingly reconsider the matter, but not until this is done.

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[1] "Measures for Avoidance of Flies, Mosquitoes, Lice and other Vermin.' H. Maxwell Lefroy, M.A., F.Z.S.

John Bale, Sons & Danielsson, Ltd., 83-91, Great Titchfield Street, London, W. 1.

No 7

THE EFFECT OF EXPOSURE TO TEMPERATURES AT OR ABOVE 100° C. UPON THE SUBSTANCE (VITAMINE) WHOSE DEFICIENCY IN A DIET CAUSES POLYNEURITIS IN BIRDS AND BERI-BERI IN MAN.

BY

HARRIETTE CHICK AND E. MARGARET HUME.

[*Reprinted from the* PROCEEDINGS OF THE ROYAL SOCIETY, B. Vol. 90]

The Effect of Exposure to Temperatures at or above 100° *C. upon the Substance (Vitamine) whose Deficiency in a Diet causes Polyneuritis in Birds and Beri-Beri in Man.*

By HARRIETTE CHICK and E. MARGARET HUME.

(Communicated by Dr. C. J. Martin, F.R.S. Received January 10, 1917.)

(From the Lister Institute.)

The influence of exposure to high temperatures upon the "vitamine," whose deficiency in a diet causes polyneuritis in birds and beri-beri in man, is a subject of great practical importance, chiefly for two reasons. In the first place, it is very necessary to know what degree of destruction, if any, is suffered by the vitamines contained in ordinary foodstuffs during the usual cooking processes, at or about 100° C. In the second place, there is the case of preserved foods, such as tinned meat, vegetables, milk, etc., which are sterilised at temperatures above 100° C. in the process of their manufacture.

The fate of the vitamines during this treatment becomes a vital matter where preserved foods form the staple article of a diet, as may be the case with troops on active service, separated from fresh food supplies.

In such circumstances the ration is usually completed with bread or biscuit, and we have made a special investigation of the wheat-grain and its various constituents in order to gauge the value of the bread or biscuit in such a diet. The results of these experiments are given in a separate communication* which contains references to the general literature of beri-beri, and to which the reader is also referred for a detailed description of the methods employed in the present work.

Polyneuritis of birds has been accepted as the complete analogy of beri-beri in man and, for the sake of brevity, we shall refer to the substance whose absence in a diet occasions polyneuritis in birds, or beri-beri in man, as the "anti-neuritic vitamine" (or anti-beri-beri vitamine). As regards the influence of high temperatures upon this substance, a great number of isolated observations exists in the literature, but no one hitherto seems to have attempted any systematic study of the point. Grijns (1901) showed that 1–2 hours' exposure to 120° C. destroyed the protective properties against polyneuritis of unmilled rice, "Katjang idjo" beans, and buffalo meat. Eijkman (1906) confirmed Grijns' results as far as unmilled rice was concerned, but did not succeed in destroying the anti-neuritic properties of horse-flesh by heating for two hours at 120° C. Schaumann (1910), however, induced polyneuritis in dogs with a diet of horse-flesh heated 1–3 hours at 120°–130° C., and Holst (1907) traced a loss of anti-neuritic vitamine in beef after heating to 110° C. for half-an-hour; this loss was much more marked after one hour at 120° C. In the case of dried peas or unpeeled barley, on the other hand, kept for half-an-hour at 115° C., no particular damage was done. Most observers have not detected any destruction of vitamine at 100° C.

In these and other experiments, the temperatures noted appear to be those registered by the autoclave or steamer used, no measurement being apparently made in the interior of the substance heated. This is an important point, as the latter temperature remains for a surprisingly long time far below the former (see Table I), especially if the material investigated has a low conductivity, which is true of most foodstuffs, especially if fairly dry. Further, no attempt has been made to investigate the time relations of the destruction process of the vitamine. This is obviously an exceedingly difficult problem, seeing that the only available instrument for measurement is so very imperfect. Nevertheless it seemed worth while to make the following effort.

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* The preceding paper.

Table I.—Curative Experiments showing the Influence of High Temperatures upon the Anti-neuritic (Anti-beri-beri) "Vitamine" contained in Wheat Embryo. Sample B, containing about 15 per cent. fine Bran. Water Content 11 to 14 per cent. Heating done in a steam autoclave. Material administered by the mouth as boluses made with a little water.

No. of expt.	Treatment of material.	Temperatures reached.	Time of heating.	Water content of material.	No. of bird.	Amount of dose given.		Result.	No. of days cure lasted.	Nature of symptoms.	Time of sickening.
						In terms of food-stuff.	In terms of dry weight.†				
		° C.	mins.	per cent.		grm.	grm.				days.
1*	Not heated, control birds	—	—	11	147	1·0	0·9	Improvement	3	Lameness	23
					161	1·0	0·9	Complete cure	5	Head symptoms	14
					162	1·0	0·9	No improvement, died	—	Lameness and crop-bound	17
					220	1·0	0·9	Died 24 hours later	—	Head symptoms and lameness	23
					221	1·0	0·9	Died 48 hours later	—	Lameness, head symptoms and crop-bound	23
					131	2·5	2·2	Complete cure	9	Lameness and head symptoms	24
					164	2·5	2·2	Very slight improvement for 24 hours	—	Lameness and crop-bound	26
					232	2·5	2·2	Died	—	Head symptoms	12
					234	2·5	2·2	Complete cure	9	Head symptoms	18
					236	2·5	2·2	Complete cure	8	Head symptoms	22
2	80 grm. placed in steamer at 100° C. for 60 mins.	90–100	40	13	132	2·5	2·2	Improvement	4	Lameness	31
					136	2·5	2·2	Incomplete cure	about 4	Head symptoms	21
					141	2·5	2·2	Complete cure	6	Head symptoms	18
					235	2·5	2·2	Cure	8	Lameness and head symptoms	17
					237	2·5	2·2	Died in 12 hours.	—	Lameness and head symptoms	18

3	40 grm. placed in steamer for 140 mins.	98–103	120	12	178	2·5	2·2	Complete cure	7	Head symptoms	14
					222	2·5	2·2	Incomplete cure	5	Head symptoms	22
					223	2·5	2·2	Complete cure	6	Head symptoms	22
					247	2·5	2·2	Complete cure	6	Head symptoms	18
					244	2·5	2·2	Great improvement	7	Lameness and head symptoms	18
4	100 grm. placed in autoclave at 113° C. for 60 mins.	102–107	40	12·2	142	2·5	2·2	Improvement	3	Lameness and head symptoms	18
					146	2·5	2·2	Complete cure	7	Head symptoms	18
					245	2·5	2·2	Incomplete cure	5	Lameness and head symptoms	21
					258	2·5	2·2	Cure	4	Head symptoms	18
					278	2·5	2·2	Cure	5	Head symptoms	15
5	100 grm. placed in autoclave at 122° C. for 60 mins.	110–117	40	14·4	160	2·5	2·1	No improvement, died in 48 hours	—	Lameness and crop-bound	16
					186	2·5	2·1	Very slight improvement	2	Lameness	10
					214	2·5	2·1	Great improvement, not cured	4	Head symptoms	20
					259	2·5	2·1	Died in 48 hours	—	Head symptoms	14
					276	2·5	2·1	Improvement, not a cure	4	Lameness and head symptoms	16
					145	5·0	4·3	Cure	10	Head symptoms	21
					246	5·0	4·3	Complete cure	5	Head symptoms	21
					256	5·0	4·3	Improvement	3	Head symptoms	15
					262	5·0	4·3	Improvement	7	Head symptoms and lameness	30
					257	5·0	4·3	Incomplete cure	4	Head symptoms	32
6	50 grm. placed in autoclave at 122° C. for 150 mins.	118–124	120	12·4	229	5·0	4·4	Improvement	5	Head symptoms	19
					230	5·0	4·4	Improvement	5	Lameness and head symptoms	19
					228	10·0	8·8	Slight improvement	5	Head symptoms	27
					281	10·0	8·8	Slight improvement	3	Head symptoms	17

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* The results of Experiment 1 have been used previously (see preceding paper) to institute a comparison between the curative values of wheat-germ and wheat-bran.

† After heating to 100–110° C.

Wheat embryo, in which the anti-beri-beri experiment is highly concentrated, was selected as suitable material for the present experiments, and 50–100 grm. were heated in a steam autoclave at different temperatures for different intervals of time. It was found that the interior of the material very slowly acquired the temperature of its surroundings, and special steps were taken to ascertain the exact temperatures experienced. In cases where the material was steamed at 100° C. (Expts. 2 and 3, Table I) the real internal temperature was measured by means of a maximum thermometer, whose bulb was inserted in the middle of the mass to be heated, and whose stem projected through the neck of the bottle containing it. The whole was lifted from the steamer for an instant at intervals of about 20 minutes, in order to obtain readings from the thermometer and to construct a curve of rise of temperature. In case of temperatures above 100° C. this procedure is not possible, and it was necessary to make special control experiments in which similar amounts of similar material were placed under exactly the same conditions as those of the actual test. Steam was blown off after various intervals of time from the start, the autoclave opened and note taken of the maximum temperatures registered both inside the vessel containing the wheat germ, and outside in the autoclave. By means of a series of such tests made separately, a temperature curve was constructed from which an accurate estimate could be made of the temperatures actually experienced by the material exposed. The data upon these points are set forth in the first three columns of Table I, above.

The experiments fall into two classes (*a*) those at or near 100° C., devised to be applicable to the ordinary processes of cooking, and (*b*) those above 100° C., intended to give information as to what may be expected to happen during sterilisation of tinned and canned foods. The effect on the properties of the wheat germ of exposure to these temperatures was studied by the following method. Comparison was made of the minimum doses of (*a*) original and (*b*) heated germ required to cure pigeons (of weight 300–400 grm.) acutely ill with polyneuritis, brought on by an exclusive diet of polished rice. From the results given in Table I it is seen that destruction of the anti-neuritic vitamine progresses very slowly at 100° C. or thereabouts. In fact, after two hours' exposure to such a temperature (Expt. 3) our experiments fail to reveal any significant loss in anti-neuritic properties—the curative dose of the heated "germ" remaining about 2·5 grm., the same as that of the unheated material (Expt. 1). After exposure for 40 minutes to a mean temperature of about 113° C. the efficiency, compared with the unheated control, is reduced to about one-half, and after subjection to 118°–124° C. for two hours, to something less than a quarter, possibly to one-tenth. The results in this series reveal all the imperfections alluded to elsewhere (see preceding paper) by the authors as peculiar to the curative type of experiment.

The material used in these experiments had rather a low water content, 10 per cent., and the vitamine present might be supposed to be protected from destruction on that score. A similar set of data are now being obtained, using a soluble yeast extract, containing over 60 per cent. water, whose vitamine content, reckoned on dry weight, is of about the same order as that of wheat germ. These experiments are not yet complete, but enough results have been already obtained to show that the extra water present during the heating has not enhanced at all the destructive effect of high temperatures upon the active substance.

It is therefore justifiable to apply the results obtained above with pure wheat germ to such processes as the baking of bread, biscuit, etc., where the water content may approach 50 per cent. The time taken to bake an average loaf does not exceed one hour, and the internal temperature rises to about 101° C. We may therefore conclude that, if wheat germ is included in the flour from which the bread is made, its anti-neuritic (anti-beri-beri) properties will survive the baking process, suffering very little damage.

The rapid destruction of anti-neuritic vitamine in the neighbourhood of 120° C. suggests that many tinned foods will be found deficient in respect of this constituent, and such we have found to be the case. We have examined two samples of army " meat and vegetable ration." In order to concentrate any vitamines they might contain, we extracted the air-dried material with alcohol (see Cooper, 1913, and H. C. and E. M. H., *loc. cit.*, p. 46), taking up the residue, after evaporating the alcohol under reduced pressure, with a small amount of water. In the case of Ration A, a very slight improvement in symptoms, but nothing approaching a cure, was obtained after administration of an extract equivalent to 350 grm. (112 grm. dry weight), and in case of Ration B, an incomplete cure after the equivalent of 440 grm. (106 grm. dry weight). Working with fresh meat, and using a similar method, Cooper (1913) obtained cures with the equivalent of 140 grm. (= 30 grm. dry weight). In the two preserved " rations " there would therefore appear to have been extensive destruction of anti-beri-beri properties during the process of manufacture.

Summary and Conclusions.

1. Exposure of wheat embryo to a temperature of about 100° C. for two hours resulted in no significant loss in anti-neuritic " vitamine." If, therefore, it is included in the flour from which bread or biscuit is made, it can be relied upon to retain its anti-neuritic properties after baking.

Table II.—Influence of High Temperatures upon the Anti-neuritic (Anti-beri-beri) "Vitamine" contained in Yeast Extract (Sample A, soluble; Water Content 65 per cent.). Curative experiments upon pigeons (300–400 grm.) suffering from acute polyneuritis, induced by an exclusive diet of polished rice. Material administered directly into the crop by means of a syringe with blunt nozzle. Heating done in a steam autoclave.

No. of expt.	Temperature.	Time of heating.	No. of bird.	Amount of dose given.		Result.	Time taken by the bird to sicken.	Nature of symptoms.	Time the cure lasted.
				In c.c. of extract.	In grm. dry weight.				
	° C.	mins.					days		days
1	Not heated,	control	294	2	0·7	No improvement, died in 3–4 hours	15	Sudden and very severe	—
			296	2	0·7	No improvement, died	21	Lameness and crop symptoms	—
			269	2	0·7	Great improvement	—	Lameness and head symptoms	3
			190	2	0·7	Incomplete cure	43	Lameness	6
			215	2	0·7	Complete cure	19	Head symptoms	6
			313	3	1·0	Improvement	12	Lameness	6
			200	3	1·0	Cure	17	Head symptoms	6
			201	3	1·0	Cure	17	Lameness	7
			293	4	1·4	Died	28	Bad case, crop involved	—
			192	4	1·4	Cure	22	Lameness and head symptoms	9
			189	4	1·4	Complete cure	15	Head symptoms	6
			193	4	1·4	Complete cure	24	Head symptoms	11
			299	4	1·4	Complete cure	20	Lameness	9
2	100	60	184		1·4	Died	21	Lameness, head and severe crop symptoms	—
			191	4	1·4	Improvement	14	Lameness and head symptoms	5
			187	4	1·4	Incomplete cure	25	Lameness	11
			188	4	1·4	Incomplete cure	25	Lameness and head symptoms	8
			185	4	1·4	Cure	21	Lameness and head symptoms	5

			280	6	2·1	Incomplete cure	18	Head symptoms	9
			303	6	2·1	Incomplete cure	21	Lameness and head symptoms	7
			203	6	2·1	Cure	22	Lameness and head symptoms	11
			227	6	2·1	Complete cure	13	Lameness and head symptoms	8
			301	6	2·1	Complete cure	16	Head symptoms	8
3	122	60	308	4	1·4	Improvement, but died after three days	30	Lameness and head symptoms	3
			306	4	1·4	Great improvement	25	Head symptoms	5
			307	4	1·4	Complete cure	19	Lameness and head symptoms	7
			194	5	1·7	Cure	15	Lameness	5
			195	5	1·7	Cure	21	Head symptoms	7
			163	6	2·1	Died	20	Lameness and crop symptoms	—
			305	6	2·1	Died	22	Lameness	—
			202	6	2·1	Cure	22	Lameness	7
			213	6	2·1	Cure	16	Head symptoms	5
4	122	120	319	6	2·1	Died in 12 hours	20	Head symptoms	—
			317	6	2·1	Died in 12 hours	20	Lameness and head symptoms	—
			310	6	2·1	Great improvement	31	Lameness and head symptoms	5
			312	6	2·1	Great improvement	30	Lameness	6
			297	6	2·1	Incomplete cure	—	Head symptoms	4
			322	10	3·5	Died in 24 hours	17	Head symptoms	—
			311	10	3·5	Cure	16	Lameness	5
			316	10	3·5	Cure	21	Head symptoms	9
			315	10	3·5	Complete cure	20	Lameness and head symptoms	8

2. At temperatures in the neighbourhood of 120° C., however, there was a swift destruction of anti-neuritic properties. This fact has an important bearing where diets are largely composed of preserved and tinned foods previously sterilised at temperatures above 100° C.

[*Addendum received August* 2, 1917.—Since the above paper was written the experiments with yeast extract alluded to on p. 65 have been completed and the results set forth in Table II, above.

The material used was a soluble yeast extract, made up to a solution of convenient strength; it contained about 35 per cent. of solids and 65 per cent. of water. As before, the heating was done in a steam autoclave; the solutions to be heated were placed in large-sized test-tubes, in quantities not exceeding 100 c.c. With this volume of liquid it was found that there was no significant delay in acquiring the temperature of the autoclave, if the test tube were placed in position while the latter was still cool.

The results obtained offer an interesting comparison with those of Table I above, where the substance heated (wheat germ) was comparatively dry (water content 10 to 14 per cent.). When due allowance is made for the large margin of error inherent in this type of experiment (see preceding paper, and p. 65, above), the results of Tables I and II are found to be in reasonable accord with one another. After exposure to 100° C. for one hour, slight loss of vitamine is evident in the yeast extract, a greater loss apparently than took place with the wheat embryo at this temperature, see Table I, Expts. 2 and 3. This may possibly be due to the excess of water contained in the extract. In the neighbourhood of 120° C. destruction of the vitamine was rapid in both cases.]

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N° 8

THE DISTRIBUTION IN WHEAT, RICE, AND MAIZE GRAINS OF THE SUBSTANCE, THE DEFICIENCY OF WHICH IN A DIET CAUSES POLYNEURITIS IN BIRDS AND BERI-BERI IN MAN.

BY

HARRIETTE CHICK AND E. MARGARET HUME.

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The Distribution in Wheat, Rice, and Maize Grains of the Substance, the Deficiency of which in a Diet causes Polyneuritis in Birds and Beri-beri in Man.

By HARRIETTE CHICK and E. MARGARET HUME.

(Communicated by Dr. C. J. Martin, F.R.S. Received January 10, 1917.)

(From the Lister Institute.)

Our attention was turned to this subject by the reported occurrence of beri-beri among our forces in the Dardanelles and Mesopotamia during the autumn and winter of 1915 (see Wilcox, 1916, I and II). Owing to the exigencies of the military situation, many individuals in these regions subsisted for considerable periods mainly on tinned meat, jam and white bread (or biscuit baked principally from white flour). Beri-beri has in recent years been included among the deficiency diseases, and there was therefore good presumptive evidence that the diet had been at fault. We examined two samples of tinned meat-and-vegetable ration by the methods described below, and found, as was to be expected, that the substances preventing beri-beri in the fresh materials had not survived the heat-sterilisation undergone in the process of canning. The case of bread and biscuit demanded a more extended investigation, and the present work is concerned with the nutritive properties of cereals generally, especially wheat.

One member of this group, viz., rice, has been exhaustively studied in recent years, and the work, among others, of Eijkman, Grijns, and Braddon has established the etiology of tropical beri-beri in a deficiency in the diet of rice-eating people, caused by the removal of certain materials from the grain during milling. The presence in the rice-bran or rice polishings of a substance essential for nutrition has been demonstrated by many observers, including those named above, and also by Schaumann (1910), to whom and to Funk (1913) the reader is referred for a complete bibliography upon the subject. Funk (1912) adopted the term "vitamine" for this essential material, and for the sake of brevity we shall use the expression "anti-neuritic vitamine"* to express the substance whose absence in a diet causes beri-beri. The vitamine in the rice grain was supposed to be contained in the layer of cells rich in protein (aleurone-layer) situated immediately underneath the pericarp of the husked grain. These cells form the outer layer of the endosperm, but are removed with the bran during milling.

As a result of experiments made chiefly with wheat, but also including rice and maize for purposes of comparison, we have reached the conclusion that the more important receptacle of the anti-beri-beri vitamine is the embryo or germ, and not the pericarp, of the grain. In the case of wheat, there are already some facts in the literature which indicate such a conclusion, but the exact composition of the materials employed in those researches was not sufficiently controlled for any dogmatic conclusion to be drawn. For example, Holst (1907) and Edie and Simpson (1911) have shown that a diet of white bread (milled wheat-endosperm) will induce polyneuritis (beri-beri) in pigeons in the same manner as polished rice (milled rice-endosperm), and the latter observers proved that birds would remain well and healthy if (*a*) whole-meal bread or (*b*) "standard" bread is used instead. The last-named article, however, in spite of its title, has no standard composition; it is generally made from white flour containing an addition of wheat germ and comparatively little bran. Hill and Flack (1911) demonstrated the inferior nutritive properties of white bread compared with "whole-meal" or "standard" bread in the case of young rats. They further showed that satisfactory growth could be induced if a proportion of wheat germ were added to a diet of white bread, and it was at Dr. Flack's suggestion that we turned our attention to wheat germ in the hope that the subject would repay study. Quite recently McCollum and Davis (1915) have found that young rats will thrive upon a diet of polished rice, butter fat and salts to which either wheat embryo or rice polishings are added; they have suggested that the curative value of rice polishings for polyneuritis of birds might be attributed to the rice embryo removed with the pericarp rather than to the bran itself.

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* This expression is also convenient to mark a distinction from those substances equally essential in a human diet, for the prevention of scurvy, which may be referred to as "anti-scorbutic vitamines."

The modern "roller" milling of wheat effects so perfect a separation of the constituent parts of the grain that trustworthy material for experiment is readily obtained. During the "break" of the grain by the rollers the pericarp, being brittle, is broken up into bran, while the embryo, being soft and oily, is squeezed out flat. The subsequent operations of milling, which include such processes as sieving, fanning and centrifuging, lead to a complete separation of these two products. With rice, however, this is not the case, and the few experiments we made with rice germ were made with materials separated by hand in the laboratory.

Methods Employed.

Polyneuritis of birds has been accepted as analogous to beri-beri in human beings with regard to etiology, symptoms, and methods of cure. The following experiments were, therefore, made with pigeons of 300–400 grm. weight, and were of two kinds, the methods adopted being in principle those used by Cooper (1913 and 1914), working in this Institute.

(1) *Preventive,* in which determination was made of the minimum amounts of the various materials in question, that must be added to a (relatively) vitamine-free diet in order to prevent the occurrence of polyneuritis. Polished rice, about 40 grm. daily, formed the vitamine-free diet, and prevention was considered to be successfully accomplished if the bird showed no symptoms of polyneuritis after a period of about 60 days. Unprotected birds usually developed symptoms in 15–25 days.

(2) *Curative,* in which determination was made of the minimal amount which, administered by the mouth, would cure a bird suffering from acute polyneuritis. A bird in this condition, brought on by an exclusive diet of polished rice, will usually die within 24–48 hours if not treated. In order to be able to administer the requisite amount of curative substance in time Cooper (*loc. cit.*) concentrated the vitamines by extracting the air-dried material with absolute alcohol in the cold and evaporating the alcoholic extract to dryness under reduced pressure. This residue was taken up in a small volume of water and definite quantities were given by the mouth, the doses being reckoned in terms of the original foodstuffs. We found it possible, however, in most cases, to give the curative doses of the original materials without preliminary extraction. By this means we gained a better idea of their absolute vitamine value in the natural condition, and avoided the loss due to the extraction processes. Our method, however, has its drawbacks, as, unless the crop of the bird is fairly empty at the time when a cure is essayed, digestion and absorption will be slow and difficult. In addition, these processes are frequently interfered with by the paralysis of the bird, so that cures are often slow (24 hours to 3 or 4 days) in progress, and it is unusual

to see the swift dramatic changes (in 6–24 hours) so typical of the cure of polyneuritis in birds, when the vitamine is administered in a soluble form. Nevertheless, with the exception of a small proportion of cases, where the character of the symptoms and the general condition were unsuitable, cures can usually be accomplished if the amount of vitamine given is adequate. Some examples are given in Tables V and VI below, in which similar material was used for cures both before and after concentration of the active substance by extraction. In the case of wheat germ, the curative dose was found to be equal to 2·5 grm. of the original material ; after extraction, the equivalent of 15 grm. was required, which result shows the large proportion of vitamine lost by the extraction process.

In the case of comparative curative experiments the difficulty is, firstly, to form a judgment of the severity of the symptoms, and, secondly, to obtain a sufficiency of acute cases in which this shall be approximately equal. This difficulty was not altogether overcome, as will be seen in the somewhat irregular results of the experiments described below, especially those set forth in Table VI. Preventive experiments form the more satisfactory method of estimating the vitamine-content in foodstuffs and yield much more regular results. The drawback is that they take at least two months to complete, and, if adopted exclusively, would limit the scope of any enquiry.

Feeding was artificial in both cases and the rice rations given in the Tables must be regarded as approximate only, since the birds frequently vomited small amounts after feeding. In case of the wheat-bran and wheat-germ rations, no significant amount of vomiting was noticed, nor was the rice vomited when these substances were added to the diet.

Wheat Embryo.—Tables I and II give the results of preventive experiments with two different samples of wheat germ, and in each case a series of birds was arranged to act as control, with a diet consisting exclusively of polished rice. The results of these two sets of experiments are in concordance ; in both a ration of 3 grm. wheat germ, given every second day, was found to afford complete protection from polyneuritis. With 2 grm. every second day, half the birds used in each experiment were protected.

Wheat Bran.—The wheat bran used for comparison with the wheat germ was what is known as "Broad bran," and the sample R.I. (Table III) was obtained from the same wheat as germ R.I. (Table II) and white flour R.I. (Table IV). It consisted of large particles, and in its thickness comprised all the outer layers of the grain. Traces of the starchy endosperm were visible upon the inside surface, and the presence of the aleurone-layer was demonstrated by sections. The sample R.I. was obtained from a modern

Table I.—Minimum Ration of Wheat Germ which must be added every Second Day to a Diet of Polished Rice in order to prevent Onset of Polyneuritis in Pigeons (300–400 grm.). Sample A; Water Content 13 per cent.

Experiment No.	Germ ration every second day.	Rice ration daily.	No. of bird.	Time elapsing before onset of polyneuritis.		Weight changes.				Remarks.	
						Original weight of bird.	Final weight of bird.	Change in weight during period of experiment.			
	grm.	grm.		days.	mean, days.	grm.	grm.	per cent.	mean, per cent.		
Control	0	About 40	81	18		329	284	−14			
			82	15		295	290	− 2			
			83	12		285	237	−17			
			84	15		355	337	− 5			
			85	26		280	234	−16			
			86	13		279	259	− 7			
			87	23		384	289	−25			
			88	13	17	319	284	−11	−12		
1	0·5	30–40	70	26		400	284	−29		No protection.	
			71	25	25·5	385	324	−16	−22	No protection.	
2	1·0	30–40	72	17		354	279	−21		No protection.	
			76	28	22	393	342	−13	−17	No protection.	
3	2·0	30–40	74	More than 67		352	272	−23		Two birds protected out of four	—
			75	57–67		345	277	−20			Slight chronic symptoms after 57th day.
			95	31		385	330	−14			—
			96	More than 63		312	290	− 7	−16		Remained without symptoms for 8 days after discontinuing germ ration.
4	3·0	30–40	92	More than 63		307	280	− 9		All birds protected	Remained without symptoms for 11 days after discontinuing germ ration.
			93	More than 63		312	312	0			Remained without symptoms for 16 days after discontinuing germ ration.
			94	More than 63		310	304	− 2	− 4		Remained without symptoms for 18 days after discontinuing germ ration.
5	4·0*	30–35	77	More than 63		370	429	+16			Remained without symptoms for 8 days after discontinuing germ ration.
			78	More than 63		292	310	+ 6	+11		Remained without symptoms for 10 days after discontinuing germ ration.
6	6·0*	30–35	79	More than 63		367	460	+25			Remained without symptoms for 15 days after discontinuing germ ration.
			80	More than 63		385	522	+36	+30		Remained without symptoms for 12 days after discontinuing germ ration.

* These rations were given as 2 grm. and 3 grm. daily, respectively, for purposes of convenience; 6·0 grm. germ is an excessive meal for a bird of this size.

Table II.—Minimum Ration of Wheat Germ which must be added every Second Day to a Diet of Polished Rice in order to prevent Onset of Polyneuritis in Pigeons (300–400 grm.). Sample R.I.; Water Content 16 per cent.

Experiment No.	Germ ration every second day.	Rice ration daily.	No. of bird.	Time elapsing before onset of polyneuritis.		Weight of bird.		Change in weight during experiment.		Remarks.
						Original.	Final.			
	grm.	grm.		days.	mean, days.	grm.	grm.	per cent.	mean, per cent.	
Control I	0	About 40	160	16		305	285	− 7		
			161	14		380	332	−13		
			162	17		342	319	− 7		
			163	21		322	277	−14		
			164	26		362	288	−20		
			165	13	18	315	306	− 3	−11	
1	1·5	30–40	166	40		369	290	−21		Some degree of protection, mean time of onset of polyneuritis delayed about two weeks.
			167	32		340	299	−12		
			168	49		360	244	−32		
			169	20	35	397	321	−19	−21	
2	2·0	30–40	170	More than 63		312	250	−20		Two birds protected out of four Protection not complete, slight chronic lameness during latter part of experiment.
			171	44		362	354	− 2		—
			172	More than 63		390	360	− 8		Remained without symptoms for 5 days after discontinuing germ ration.
			173	26		395	355	−10	−10	—
Control II	0	About 40	200	17		387	298	−23		
			201	17		332	312	− 6		
			202	22		374	282	−25		
			203	22	19·5	385	297	−23	−19	
3	3·0	30–40	196	More than 63		320	330	+ 3		Complete protection Remained without symptoms for 7 days after discontinuing germ ration.
			197	More than 63		345	382	+11		Remained without symptoms for 8 days after discontinuing germ ration.
			198	More than 63		397	380	− 4		Remained without symptoms for 7 days after discontinuing germ ration.
			198	More than 63		347	422	+22	+ 8	Remained without symptoms for 8 days after discontinuing germ ration.

Table III.—Influence of Regular Ration of Wheat Bran ("Broad Bran") upon Onset of Polyneuritis in Pigeons (300–400 grm. weight) fed upon an Exclusive Diet of Polished Rice.

Description of sample.	Bran ration every second day.	Rice ration daily.	No. of bird.	Time elapsing before onset of polyneuritis.		Weight of bird.		Change in weight during period of experiment.		Remarks.
						Original.	Final.			
	grm.	grm.		days.	mean, days.	grm.	grm.	per cent.	mean, per cent.	
"R.I." (corresponds to germ "R.I." and white flour "R.I.") free from germ, roller-milled, water content 12 per cent.	0	About 40	212	16		490	365	−26		
			213	16		380	317	−17		
			214	20		432	340	−21		
			215	19	18	460	350	−24	−22	
	3·0	30–40	216	20		465	410	−12		No protection, mean time of sickening approximately equal to control.
			217	18		348	372	+ 7		
			218	26		490	360	−27		
			219	14	19·5	336	288	−14	−11·5	
	0	About 40	244	18		465	396	−15		
			245	21		493	425	−14		
			246	21		400	295	−26		
			247	18	19·5	410	316	−23	−19·5	
	5·0	30–40	248	51		375	285	−24		Small degree of protection, mean time of onset of polyneuritis delayed about two weeks.
			249	23		415	330	−20		
			250	27		398	385	− 3		
			251	40	35·5	429	352	−18	−16	
Sample A, stone-milled, probably not free from germ	0	About 40	178	14		260	225	−13		
			179	26	20	325	272	−16	−14·5	
	5·0*	30–40	174	47		324	322	− 1		Some degree of protection, time of onset of polyneuritis delayed about four weeks.
			175	48	48	284	265	− 7	− 4	
	10·0*	30–40	177	More than 63		246	230	− 6		Protection.

* Ration given as 2·5 grm. and 5 grm. daily, respectively, owing to the bulky nature of the material.

roller-mill, and may be regarded as free from germ. Birds receiving a ration of 3 grm. every second day developed polyneuritis in the same time as those upon an exclusive polished rice diet; with 5 grm. every second day the onset of symptoms was postponed for about two weeks. If the results of Table III be compared with Table II one may regard the anti-neuritic value of 5 grm. of broad bran as equivalent to 1·5 grm. germ.

On general grounds it would seem not unlikely that what value the bran possesses must be attributed to the aleurone-layer, which forms only a small proportion of the whole. Hamill (1911) states that the aleurone-layer forms about 4 per cent. of the grain and the pericarp about 15 per cent. He gives no authority for these figures, and it is difficult to see that they can be other than approximate. On this basis, the aleurone-layer would constitute about one-fifth of the bran, and, weight for weight, be about equal to the embryo as regards vitamine-content.

It is interesting to note the greater anti-neuritic power of the broad bran derived from stone-milled wheat (see Sample A, Table III). In this case no separation of the germ is effected in the milling, and each constituent issuing from the mill contains some of it.

Table IV.—Influence of a Diet of "Straight Run" White Wheat Flour in Comparison with a Diet of Polished Rice. Sample of Flour "R.I.," roller milled, free from germ and bran, from same wheat as Germ R.I. and Broad Bran R.I.

White flour ration, daily.	Polished rice ration, daily.	No. of bird.	Time elapsing before onset of polyneuritis.		Weight of bird. Original.	Weight of bird. Final.	Change in weight during period of experiment.	
grm.	grm.		days.	mean, days.	grm.	grm.	per cent.	mean, per cent.
0	About 40	256	15		416	387	− 7	
		257	32		450	345	−23	
		258	18		455	370	−19	
		259	14		449	372	−17	
		262	30	22	507	365	−28	−19
20–30	0	260	16		506	465	− 8	
		261	28		515	402	−22	
		263	13	19	485	435	−10	−13

Wheat Endosperm.—In Table IV are given the results on pigeons of an exclusive diet of "straight run" white flour, or starchy wheat endosperm (after removal of the aleurone-layer), the sample employed, viz., R.I., corresponding to bran R.I. (Table III) and germ R.I. (Table II). The birds received 20–30 grm. daily, made into pills after mixing with a minimal amount of water. By experience this ration was found to be as much as they could assimilate. Polyneuritis occurred in each instance, and the time of onset was, if anything, rather earlier than that occurring with a series of control birds fed on polished rice. There was also no significant difference in the loss of weight suffered by the birds in the two series of experiments.

Curative Experiments.

In Table V are given the results of some curative experiments made with (1) wheat germ, and (2) wheat "middlings," or the finely ground bran, which is probably not free from admixture with germ. In both cases the vitamines were concentrated by the extraction process with alcohol described above (p. 46). The relative curative values of these two materials were found to be in the ratio of about 5·5 to 1, if we judge by the "great improvement" obtained with the extracts equivalent to 9 and 50 grm. respectively in the two instances.

It is necessary to define here what is meant by the various types of cure described in Tables V and VI. The expression "complete cure" indicates that both flight and gait were perfectly restored; "cure" that one or both remained slightly imperfect, while "incomplete cure" is used to describe a rather less satisfactory condition. The birds before treatment were acutely

Table V.—Comparison of Minimum Amounts of Wheat Germ and Wheat Bran required to cure Pigeons (300–400 grm.) suffering from Acute Polyneuritis induced by a Diet of Polished Rice. The materials were extracted with alcohol and the residues obtained, after evaporation of the alcohol, taken up with water. Doses reckoned in amounts of the original foodstuff taken.

Description of material.	No. of bird.	Amount of curative dose given.		Result.
		In terms of natural foodstuff.	In terms of dry weight.	
		grm.	grm.	
Wheat germ, Sample A, water content 13 per cent.	88	10	8·7	Great improvement.
	83	15	13·0	Complete cure.
	56	15	13·0	Complete cure.
	59	20	17·4	Complete cure.
"Middlings," or fine wheat bran, probably not free from germ, water content 11 per cent.	104	28	25	Improvement, no cure.
	114	56	50	Improvement, no cure.
	129	56	50	Great improvement.

Table VI.—Minimum Amount of Wheat Embryo required to cure Pigeons (300—400 grm. weight), suffering from Acute Polyneuritis induced by a diet of Polished Rice. Material administered by the mouth, without preliminary extraction, as boluses made with a little water.

Description of material.	No. of bird.	Amount of curative dose.		Result.	No. of days cure lasted.	Nature of symptoms.	Time of sickening.
		In terms of foodstuff.	In terms of dry weight.*				
		grm.	grm.				days.
Wheat-embryo, Sample B, containing about 15 per cent. fine bran; water content 11 per cent.	147	1·0	0·9	Improvement	3	Lameness	23
	161	1·0	0·9	Complete cure	5	Head symptoms	14
	162	1·0	0·9	No improvement, died	—	Head symptoms and crop-bound	17
	220	1·0	0·9	Died after 24 hours	—	Head symptoms and lameness	23
	221	1·0	0·9	Died after 48 hours	—	Head symptoms, lameness and crop-bound	23
	131	2·5	2·2	Complete cure	9	Head symptoms and lameness	24
	164	2·5	2·2	Very slight improvement for 24 hours	—	Lameness and crop-bound	26
	232	2·5	2·2	Died	—	Head symptoms	12
	234	2·5	2·2	Complete cure	9	Head symptoms	18
	236	2·5	2·2	Complete cure	8	Head symptoms	22

* After heating to 100–110° C.

ill, and were usually quite helpless; by the term "improvement" is meant that the acute symptoms subsided, and that the bird was out of danger for a time. It should be added, perhaps, that these expressions deal with symptoms alone, and that nothing is assumed with regard to the actual lesions. It is obviously impossible that if axon degeneration is present this can be restored in the short period occupied by the "cure." The quality of the cure was also studied by continuing the diet of polished rice and noting the time elapsing before the recurrence of polyneuritis symptoms.

Further curative experiments with wheat germ are set forth in Table VI. Here no extraction with alcohol took place, but the moistened germ was given in the form of boluses, which were readily swallowed by the bird. The results of this experiment, compared with those given in Table V, show how great is the loss of vitamine due to the extraction process, and how small, comparatively (from 1 to 2·5 grm.), is the amount of wheat germ required to effect a cure when given in the natural condition.

The results also show the degree of irregularity that must be expected in this type of experiment (see above, p. 47). This is doubtless due to differences in the nature and severity of the symptoms in the different birds, all of whom were acutely ill, and in certain cases also to difficulty in digesting and absorbing the curative material. Those cases in which the action of the crop was affected by polyneuritis are by far the hardest to cure, and probably should be disregarded in any attempt to estimate vitamine-content in the substances administered as cures.

From Table VI one may conclude that 2·5 grm. of wheat embryo may generally be relied upon to cure acute polyneuritis in a pigeon of 300–400 grm. weight; at the same time, cures were obtained, exceptionally, with 1 grm., and occasionally with 1·5 and 2 grm.

For comparison with the above, some curative experiments were made with "broad bran," this being again chosen as likely to provide a specimen of pericarp free from any admixture of germ. In one case we failed to effect a complete cure with 10 grm., and in another we obtained no improvement with 5 grm., while in a third instance we obtained a cure with 5 grm. These experiments present special difficulties as regards their correct interpretation; the bran is very bulky, the dose is large, and difficulties of absorption correspondingly enhanced. In fact, a much more trustworthy estimate of the relative vitamine values of wheat pericarp and embryo, weight for weight, are obtained when the vitamines are concentrated and administered in a soluble form as in Table V (1 : 5·5), or, better still, when the results of preventive experiments are used to make the comparison (1·5 : 5, see Tables I–III).

Rice.

We have only made a few preliminary experiments with rice, owing to the difficulty in obtaining material. Some little work has, however, been accomplished, using rice embryo separated from the unmilled grain by hand, in the laboratory. The rice embryo is extremely small, and amounts only to about 2·5 per cent. by weight of the husked grain, so that the work is exceedingly laborious; only a small amount of material was obtained, and all the experiments were perforce of the curative type. The rice-bran used for comparison was a sample kindly furnished by a firm of rice millers, and contained both pericarp and embryo. The experiments with embryo demonstrated the high concentration of vitamine in this organ; the birds showed some improvement in symptoms after administration of 0·3 grm., and better results, almost amounting to cures, after 0·5 grm., while a dose of 1 grm. gave a complete cure lasting nine days. In case of the bran, a distinct improvement was manifested on one occasion after receiving 4 and 6 grm. respectively. In one case improvement was also noticed after 5 grm., and in a second case, not so severe as the first, a complete cure was effected. Complete cures were obtained after doses of 8 and 10 grm. on more than one occasion.

Maize.

A few trials have been made with maize embryo, which also has proved to be very rich in anti-neuritic vitamine. In this cereal the embryo is comparatively large, and it was easily picked out by hand, especially if the grain were soaked in water overnight. After this treatment the embryo amounted to 15 per cent. by weight of the entire grain. In the case of maize, the plumule (shoot) and radicle (root) of the embryo can be separated with ease from the scutellum, which organ is exceedingly large, and amounts to about 12·5 per cent. of the soaked grain, while the plantlet comprises the remaining 2·5 per cent. Both parts of the embryo, however, proved to be receptacles of vitamine. Cures were achieved with 1 and 2 grm. of the wetted plantlet (equivalent to 0·6 and 0·7 grm. dry weight respectively). A complete cure, lasting 13 days, followed a dose of 2 grm. (dry weight) of the entire germ (scutellum, plumule, and radicle), and a less satisfactory result with 3 grm. (dry weight) of scutellum. Smaller quantities than these were not given, and it is hoped later to investigate this cereal further.

Relation between the Preventive Ration and the Curative Dose.

A very curious result has emerged from the foregoing experiments, viz., the very close approximation between the daily ration of wheat germ required to prevent the onset of polyneuritis, and the dose necessary to cure a bird in the acute condition. While 3 grm. must be added every second day to a diet of polished rice, to prevent polyneuritis with certainty (see Tables I and II), the curative dose is found to be only 2·5 grm., and in exceptional cases 1 grm. sufficed. These curative doses cause disappearance of polyneuritic symptoms, brought on by the exclusive diet of polished rice, and the bird is usually maintained in good health for several days, notwithstanding the deficiency in its food (see Table VI). After this period recurrence of symptoms again takes place.

This relation between the preventive ration and the curative dose is not confined to any particular anti-neuritic substance, but appears to be general. In case of a yeast-extract (water-content = 30 per cent.), the daily preventive dose was found to be from 1 to 2 grm., and the curative dose from 2 to 3 grm., occasionally 1 grm. This result is in accord with the observations of Cooper, who (1913) found that 2·5 grm. pressed yeast (dry weight = 0·5 grm.), given daily, would prevent polyneuritis in pigeons fed otherwise with polished rice. In order to cure pigeons suffering from acute polyneuritis (1914, II), he used autolysed yeast, and found it necessary to administer the equivalent of 3-6 grm. of the original pressed yeast; on a second occasion the equivalent of 1–2 grm. was sufficient. There are no other good instances available, because curative experiments have almost universally been made with material which has been extracted with alcohol to concentrate the vitamine, while, for the preventive experiments, the natural foodstuffs have been used. As pointed out above, there is a great loss of anti-neuritic material in the extraction process as usually carried out. Hence the stated amounts required for the cure of animals show an exaggerated size when reckoned on the original foodstuffs.

That the preventive and curative doses are of the same order of magnitude is perplexing, and demands further investigation. In order to obtain some light upon this point we are making a series of preventive experiments in which the total amount of vitamine is kept constant, but the dosage, *i.e.*, size and periodicity of the dose, is varied. These experiments are not yet completed, but the only interpretation which the facts seem capable of carrying at present is that:

(1) There is an urgent daily need for a small quantity of this vital substance to maintain the metabolism of the nerve tissue;

(2) In the normal condition a considerable store is available somewhere in the animal body upon which to draw; and

(3) This store becomes suddenly exhausted, but can be temporarily restored by the administration of a small quantity from outside.

Influence of a Small Regular Ration of Wheat-Embryo in Maintaining the Weight of Birds Fed on Polished Rice.

It has been shown by numerous observers that birds fed exclusively on polished rice lose consistently in weight. In our experience, the average loss of weight under these conditions varied from 11 to 22 per cent. over a mean period varying from 17 to 22 days (see control experiments, Tables I–IV). When a ration of wheat was given insufficient to prevent the onset of polyneuritis (*i.e.*, from 0·5 to 1·5 grm. every second day), this loss in weight was not exceeded, although the mean period of the experiment lasted from 22 to 35 days (see Tables I and II).

When the wheat ration was adequate for protection against polyneuritis (*i.e.*, 3 grm. every second day), in one instance (Expt. 4, Table I) the average loss of weight was negligible, viz., −4 per cent., and in a second (Expt. 3, Table II) a gain in weight was observed, viz., +8 per cent. The total weight gained by the seven birds in these two experiments was 72 grm., or a gain of about 10 grm. per bird.

In Expt. 3, Table I, and Expt. 2, Table II, where 2 grm. wheat germ was given every second day, and polyneuritis was not prevented with certainty, there was an average loss of weight of 16 and 10 per cent. respectively, or a total loss of 365 grm. among the eight birds studied, *i.e.*, 46 grm. per bird.

The average difference in weight between individuals in the above two series of experiments is therefore 56 grm. This must be referred to a difference in diet amounting to 0·5 grm. wheat germ daily over a period of about 60 days, *i.e.*, a total of 30 grm. per head. It is therefore evident that the addition to a diet of polished rice of wheat germ in amount to prevent polyneuritis has also some influence in promoting the general metabolism of the animal.

This result is in accord with the observation of Schaumann (1911), who found that if a ration of a vitamine-containing foodstuff (such as rice-bran or yeast) were adequate to prevent onset of polyneuritis in birds, when added to a diet of polished rice, loss of weight was prevented, whereas extracts of these foodstuffs prepared by extraction with acid or alcohol, sufficient to prevent polyneuritis, did not maintain weight.

Our experiments with wheat-bran offer another example of this principle. Where the bran was free from germ, as in sample R.I. (Table III), a ration of 5 grm. each second day did not prevent polyneuritis, nor did it maintain weight. In case of sample A, which was stone-milled, and presumably contained traces of germ, a ration equal to 5 grm. each second day more nearly protected a pigeon from polyneuritis, and there was no appreciable loss of weight.

Cooper (1913) came to the conclusion that the substances preventing polyneuritis and maintaining body weight were separate and might be separately distributed in natural foodstuffs. For certain of these, *e.g.* yeast, ox-heart and ox-brain, the two substances were evenly balanced and the daily ration required to prevent polyneuritis also maintained the weight of the bird. In others, *e.g.* egg-yolk, barley, lentils, this daily ration had to be increased if loss of weight were also to be prevented. In this connection our experiments in which casein was added to a diet of polished rice (set out in Table VII) are of some interest. The casein, prepared from milk by precipitation and subjected to some purification, proved to be vitamine-free, as was expected; the addition of the extra protein had no influence upon the onset of polyneuritis. With the addition to the diet of 3 grm. of this casein daily, however, the weight of the birds was certainly maintained better than was the case with the control birds fed on polished rice alone.

Table VII.—Influence of Addition of Extra Protein (Casein) to a Diet of Polished Rice, upon the Onset of Polyneuritis in Pigeons (300—400 grm. weight).

Casein ration daily.	Rice* ration daily.	Total protein given daily.	No. of bird.	Time elapsing before onset of polyneuritis.		Weight of bird.		Change in weight during period of experiment.	
						Original.	Final.		
grm.	grm.	grm.		days.	mean, days.			per cent.	mean, per cent.
0	About 35	2·4	107	19		369	292	−21	
			108	21		469	339	−28	
			109	22	21	427	395	− 8	−19
0	About 40	2·8	104	23		300	267	−11	
			105	19		359	287	−20	
			108	16	19	339	295	−13	−14
1·5	35	3·9	110	27		365	305	−17	
			111	20		365	289	−21	
			112	21		382	335	−12	
			113	18	21·5	352	300	−15	−16
3·0	35	5·4	114	25		395	310	−14	
			115	18		365	362	− 1	
			116	14		392	385	− 2	
			117	22	20	394	345	−12	− 7

* Protein content = 6·9 per cent. ('Bulletin No. 45, U.S. Department of Agriculture'; Tibble's "Foods," 1912, p. 474).

A ration of wheat germ equal to 4 grm. every second day, which is slightly in excess of that necessary to prevent polyneuritis, had a marked beneficial influence upon the general health and well-being of the birds (Expt. 5, Table I). When this allowance was further exceeded, as in Expt. 6, Table I, where 3 grm. wheat germ were given daily, the effect was even more noticeable. The increase in weight of the two birds employed amounted, in a period of two months, to 25 and 35 per cent. respectively of their original weight, and the birds displayed unusual energy and vitality. They became wilder and more quarrelsome; they could not be kept together in one cage, as was our custom, seeing that they fought one another with great vigour, if permitted. They would also resist being taken up and examined, and if the cage were opened, would usually take up a sideways position at bay, striking out with the wing against any invader. All this demeanour is in very marked contrast to the gentleness characteristic of birds which are artificially fed and subjected to the large amount of handling which is involved in this type of experiment.

Summary and Conclusions.

1. The foregoing experiments deal with the distribution of "anti-neuritic (anti-beri-beri) vitamines" in the various constituents of the wheat, maize, and rice grains. By "anti-neuritic vitamine" is meant the substance whose deficiency in a diet causes polyneuritis in pigeons and beri-beri in man.

2. Wheat-endosperm, after removal of the aleurone-layer in the ordinary milling processes, constitutes white flour. It is deficient in this vitamine, and if used as an exclusive diet will induce polyneuritis in pigeons (or beri-beri in man) in a manner identical with polished rice.

3. In both the rice and wheat grain, the anti-neuritic vitamine is concentrated mainly in the germ or embryo; it is also present to a less degree in the bran (pericarp and aleurone-layer), probably in the aleurone-layer.

4. In case of maize grain, the embryo also possesses marked anti-neuritic properties. Here the scutellum can be separated from the "plantlet" and separately investigated. Both these constituents of the embryo were found to contain anti-neuritic vitamine.

5. The practical results given under 2 and 3 show the importance of including germ in the flour from which wheaten bread or biscuit is made, especially when the diet may consist largely of preserved foods, *e.g.* tinned meats and vegetables, which are deficient in anti-beri-beri vitamines.

6. The daily ration of wheat-germ that must be added to a diet of polished rice in order to prevent the onset of polyneuritis is of the same order of magnitude as the amount which, administered by the mouth, will cure a pigeon acutely ill with polyneuritis, brought on by an exclusive diet of polished rice. This relation is not peculiar to wheat-germ but applies to other foodstuffs containing anti-neuritic vitamines, *e.g.* yeast.

7. The addition of wheat-germ to a diet of polished rice in quantity (3 grm. every second day) sufficient to prevent polyneuritis, also maintained the weight and general health of the bird. Rations in excess of this (2 grm. every day to 3 grm. every day) led to great increase in body-weight and in general well being and vitality of the birds, which, after a short period of this diet, became in remarkably fine condition.

In conclusion, our best thanks are due to Messrs. Steele and Co. (rice millers), to The Hovis Milling Co., and to Messrs. J. and H. Robinson (wheat millers), for kindness in supplying us with material for experiment, and especially to Mr. E. G. Ellis, of the last-named firm, for much valuable advice and assistance. We are also much indebted to Professor A. Harden, F.R.S., for kindly preparing one of the extracts mentioned in Table V, to Captain A. H. Osman, of the Home Forces Pigeon Service, for a generous supply of pigeons for this work, and lastly to our assistant, Mr. A. H. Robins, for his devoted work in connection with the artificial feeding and care of the experimental birds.

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XXXIV. ANAPHYLAXIS TO THE SEPARATED PROTEINS OF HORSE-SERUM.

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The earliest attempt to examine the part played by the different serum proteins in anaphylaxis to a foreign serum was made by Gay and Adler [1908]. Gay and Southard had based a theory of the anaphylactic condition on an erroneous interpretation of the phenomenon of passive anaphylaxis. This theory explained the sensitive condition as due to a remnant of the foreign serum, introduced at the sensitising injection, and still present in the system. Clearly it might be expected that the remainder thus conferring sensitiveness would belong to a different fraction of the serum protein from that which, at the second injection, acted as a poison. In accordance with this expectation, Gay and Adler found that the euglobulin fraction of a serum had great power of producing sensitiveness to the serum from which it was separated, but no toxic action when reinjected into the sensitised animal. Taking further fractions they observed a progressive decline of sensitising power, and increase of toxicity to the sensitised animal, as the proportion of ammonium sulphate needed for precipitation became greater; so that the highest fraction, the albumin, had practically no power of producing sensitiveness, but a maximum toxicity for animals sensitised with the whole serum, or with the lower fractions. These conclusions are in complete disaccord with the later results, obtained by various investigators, on anaphylaxis to pure proteins, of animal or vegetable origin. Such results have indicated a considerable degree of specificity of the reaction, not only for the species from which the protein originated, but even for pure proteins separated from an organ or tissue.

Doerr and Russ [1909, 1], repeating Gay and Adler's experiments with more attention to quantitative detail, obtained an entirely different result. Like Gay and Adler, Doerr and Russ worked with guinea-pigs, and separated the proteins of the ox or horse-serum used for sensitising by fractional precipitation with ammonium sulphate. They found, however, that sensitising power, and toxicity for the sensitised animal, declined in a parallel manner, with increase of the proportion of ammonium sulphate necessary for precipitation; so that the euglobulin fraction exhibited the maximum anaphylactic activity in either direction, the albumin being, in both respects, practically inert, and the pseudoglobulin occupying an intermediate position. Doerr and Russ's results are in conformity with the general tendency of the evidence obtained with other proteins. The only anomaly is the failure of the albumin fraction to act as an anaphylactic antigen. In a later paper [1909, 2], however, they showed a parallel failure of serum-albumin to produce a precipitin when injected into the rabbit, and, in conformity with their view of the identity of anaphylactic antibody with precipitin, a failure of serum from a rabbit so injected to confer passive anaphylaxis on the guinea-pig.

This question of the antigenic properties of the separate serum proteins, with regard to precipitin formation, has been examined by several other investigators, with results which show the widest possible variation, both in respect of the relative efficacy shown by the different proteins in exciting precipitin formation, and of the specificity to the individual proteins of the precipitins obtained. Some agree with Doerr and Russ as to the absence of a precipitin reaction to serum albumin; others, while obtaining a precipitin by injection of albumin, find that it precipitates even more strongly with globulin than with the albumin exciting its production. Doubtless a good deal of the variation is due to the varying degrees, in which the different methods employed effected a clean separation of the different proteins. The most convincing account is that given by Hunter [1905], whose paper may be consulted for the earlier literature. Hunter found that each of the serum proteins, euglobulin, pseudoglobulin, and albumin, was capable of exciting precipitin formation, and that the precipitin obtained in each case reacted most strongly with the protein used in its production, but in a modified degree with the others also; in other words, each precipitin showed a relative, or quantitative, but not an absolute, or qualitative specificity for its own protein.

Certain points in the behaviour of the serum-proteins as anaphylactic antigens seemed to deserve further investigation. Doerr and Russ's description of the progressive weakening of the anaphylactic effect in passing from the euglobulin to the albumin fraction, the pseudoglobulin being intermediate in activity, suggests a possibility that euglobulin may be the only protein in serum capable of producing the reaction, and that the weaker reaction of pseudoglobulin may be due to incomplete separation. The point is not without practical significance, as well as theoretical interest. The antibody of immune horse-serum has been shown to be associated with the pseudoglobulin fraction, and the various methods of artificial concentration have aimed at eliminating the albumin and the euglobulin; but the details of the processes in actual use would seem to ensure a more thorough exclusion of the former. It is clear, on the other hand, if the euglobulin is the anaphylactically active constituent, and the albumin indifferent in this respect, that the aim of any process for purifying antitoxin should be as thorough a removal of euglobulin as is possible in large-scale working.

METHODS.

We have confined our attention to horse-serum, specimens of the three proteins from which were available for use. Those which we used in the majority of the experiments were prepared by one of us for a chemical investigation, the results of which have already been published, together with the details of the methods of preparation [Hartley, 1914]. The euglobulin used was that prepared by Panum's method, and probably made a nearer approach to complete purity from the other proteins than did the specimens of pseudoglobulin and albumin, although these were probably as pure as repeated salting-out could make them. An additional specimen of the pseudoglobulin of horse-serum was kindly placed at our disposal by Dr H. Chick, who, after the usual separation by ammonium sulphate, had further purified it by continuous dialysing, with removal of the euglobulin thrown out of solution, during some months. Both the samples of pseudoglobulin were "euglobulin-free," in the sense that the ordinary methods of detection and separation would fail to discover euglobulin in them. But the quantity of a protein needed for sensitisation of the guinea-pig has been shown to be so minute, that it cannot be safely assumed that either was free from such traces of euglobulin as might be perceptibly effective in this direction. The same may be said with regard to a possible contamination of the albumin with minimal remnants of pseudoglobulin. In addition to the specimen above mentioned, a sample of albumin prepared in a different way was used in several experiments. This was a crystalline albumin prepared by Hopkins and Pinkus's [1898] method, and recrystallised three times, only that fraction being taken of each crop which consisted wholly of large well-formed crystals. This was dialysed free from ammonium sulphate and dried to a scale in vacuo. A portion of it was, for another purpose, dissolved in water, precipitated with cold acetone and ether, extracted for three days with warm ether, and finally taken up in water and recrystallised by Hopkins' method, yielding particularly well-formed crystals. These were again dialysed, scaled, and kept dry. Neither of these preparations showed any notable difference in action from the albumin-fraction obtained by salting-out in the ordinary way.

Most of our experiments were made on guinea-pigs, which were sensitised by hypodermic injection of a small dose, either of whole fresh serum, or of a solution of one of the separated protein preparations. After an interval, varying from 12 up to 31 days, the sensitiveness of the uterine plain muscle to two or more of the separated proteins was tested by the method previously described by one of us [Dale, 1912]. The use of this method has the definite advantage, over experiments made on a series of similarly prepared animals, that no allowance need be made for individual differences of sensitisation. The reaction of one horn of the uterus is tested with one of the pure proteins, and subsequently the reaction of the other horn, which can be regarded with some certainty as equivalent in specific sensitiveness and physiological condition to the first, is tested similarly with another of the pure proteins. Moreover the degree, to which an effective dose of either protein affects the subsequent reaction to the other, can be readily ascertained. By a careful choice of the order in which the doses are given, as much information can often be obtained from one such experiment, with regard to the relative sensitiveness to the different proteins, as would require the injection and reinjection of a long series of animals, if the more usual method of studying anaphylaxis in the guinea-pig were adopted. For purposes of control a few experiments were made by the ordinary method of intravenous reinjection into the intact animal, with observation of the degree of severity of the resulting symptoms as an index of the sensitisation. Young virgin female guinea-pigs were chosen, weighing about 180 g. at the time of the preparatory injection, so that the test of sensitisation, after an interval of two to three weeks, or more, was made when the animals weighed about 250 g. on an average.

The apparatus used was arranged, in all material particulars, similarly to that described by Dale and Laidlaw [1912]. The vessel in which the uterine horn under experiment was suspended contained, in all cases, 70 cc. of Ringer's solution. The "control" uterine horn was kept, till required, in a tube of the same solution, immersed in the same constant-temperature bath as the experimental vessel. The contents of the experimental vessel, and of the tube containing the control, were oxygenated and stirred by a stream of bubbles. The temperature employed was between 37° and 39° C. The lever recording the uterine contractions magnified twice or thrice, according to the size and activity of the uterus under observation. Similarly the weighting of the lever was varied so as to secure, in each case, efficient extension, without imposing undue resistance to contraction.

The Ringer's solution used was made up according to one or other of the formulae mentioned by one of us in an earlier paper. The results obtained were of the same kind with either, but we gathered the impression that, for keeping the general sensitiveness of the control horn at the same level as that first tested, during a long experiment lasting some hours, the solution called "Ringer I" in the earlier paper was, on the whole, the better. The various proteins tested were made up in Ringer's solution, usually in 1 % solution. The albumin and pseudoglobulin could be dissolved directly in the Ringer's solution without difficulty. Euglobulin so treated was imperfectly soluble. Although the imperfect, opalescent suspension, obtained with Ringer's solution alone, seemed to act perfectly well on the plain muscle when added to the bath, it was considered better, after the first few experiments, to use it in a more highly dispersed condition, in order to obtain a more accurate comparison of its effect with that of pseudoglobulin. With this object, the required amount was weighed out and rubbed up with a little Ringer's solution into a fine emulsion. To this was added 0·1 cc. N/10 NaOH for each 100 mgm. of euglobulin. The relatively clear solution so obtained was then diluted with Ringer's solution to a strength of 1 % euglobulin; the proportion of added soda, after such dilution, corresponding, therefore, to 1/1000 normal. Controls showed that a pure solution of soda, in this strength, when added to the bath in volume corresponding to those of the test doses of euglobulin solution, produced no noteworthy effect on the uterine activity.

A few experiments were also made on anaesthetised dogs, with record of the blood-pressure, to confirm the general applicability of some of the results obtained with guinea-pigs.

RESULTS.

Animals sensitised to whole horse-serum.

We first tested the reaction, to the pure serum proteins, of uterine muscle from guinea-pigs sensitised by a hypodermic injection of 0·01 or 0·1 cc. of whole horse-serum, given two to three weeks previously.

(*a*) *Euglobulin and Pseudoglobulin.* In two cases, out of some eighteen guinea-pigs tested, sensitisation, for some unexplained reason failed, so that the uterine muscle responded but weakly even to large doses of horse-serum. Except in these instances the plain muscle of guinea-pigs, which had received a sensitising injection of whole horse-serum, exhibited a pronounced sensitiveness to the euglobulin and pseudoglobulin of horse-serum. The sensitiveness to the two showed a general parallelism, so that, if one horn of the uterus gave a large contraction in response to 1 mgm. of euglobulin, the second horn usually reacted similarly to 1 mgm. of pseudoglobulin. Similarly, if the first horn tested gave only a small reaction with 1 mgm. of euglobulin, and a subsequent maximal reaction with 10 mgms. of the same, it was usually found that the second horn gave effects of the same order with the same doses of pseudoglobulin.

At first sight, therefore, the results might easily have been interpreted as signifying that the euglobulin and pseudoglobulin were acting as one common antigen. It will be shown later that the effects of sensitising by the separate proteins, instead of by whole serum, exclude such a supposition; but there were clear indications of the distinct antigenic properties of the two globulins, even in the experiments with whole serum. Such indications were obtained by studying the effects of desensitisation. In an earlier paper it was shown that the application of an adequate, effective dose of an antigen, to which the plain muscle has been made anaphylactic, will permanently remove its sensitiveness to that antigen, while leaving it, in other respects, practically normal. The question therefore arises, whether a dose of euglobulin, which renders indifferent to further euglobulin a preparation originally sensitive to both globulins, will at the same time remove its initial sensitiveness to pseudoglobulin, and *vice versâ.* There are three possibilities, and we have found examples of each, in our series of guinea-pigs sensitised by whole serum.

(i) The two globulins may appear to act as a common antigen, so that one horn may respond by a maximal contraction to a certain dose of euglobulin, and be thereafter insensitive not only to euglobulin, but to pseudoglobulin in like dosage; while the second horn may show an exactly parallel response to, and complete desensitisation by a like dose of pseudoglobulin. This type of reaction is illustrated by the following experimental record, Figs. 1 and 2 reproducing the corresponding tracings.

Guinea-pig No. 9. Sensitised by hypodermic injection of 0·1 cc. of whole horse-serum 16 days previously. Bath-volume 70 cc. Ringer's solution changed after the effect of each dose was complete.

1*st horn of uterus* (Fig. 1).

1 mgm. of euglobulin. Moderate contraction.
10 mgm. of euglobulin. Maximal contraction.
10 mgm. of pseudoglobulin. Nil.

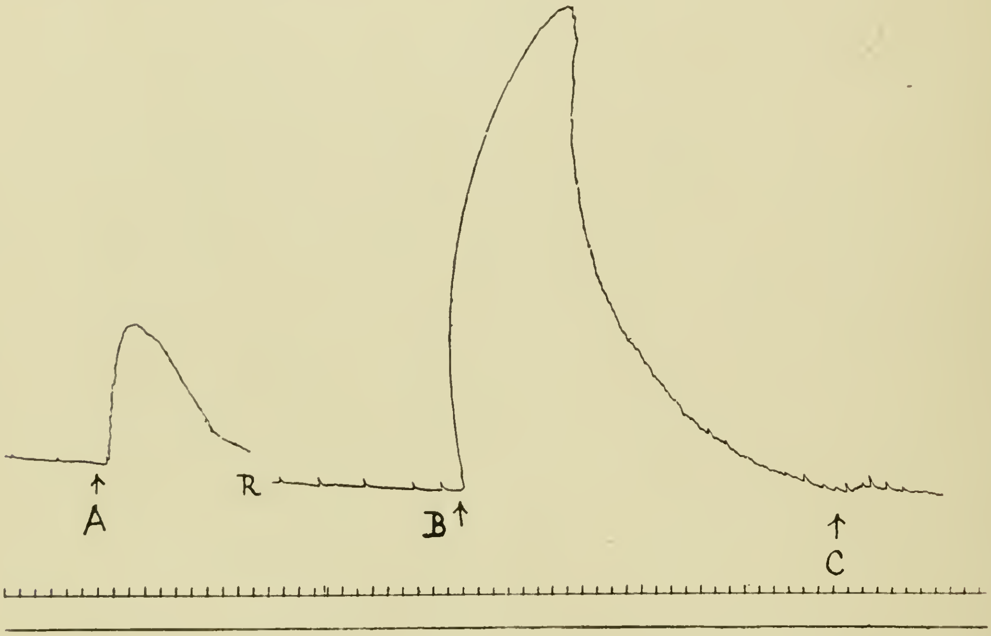

Fig. 1. Guinea-pig sensitised with 0·1 cc. of whole horse-serum. 16th day. 1st horn A. 1 mgm. of euglobulin. B. 10 mgm. of euglobulin. C. 10 mgm. of pseudoglobulin. Time marker (in all figures) marks intervals of 30 seconds. R (in all figures) indicates change of Ringer's solution.

2*nd horn of uterus* (Fig. 2).

1 mgm. of euglobulin. Moderate contraction.
10 mgm. of pseudoglobulin. Maximal contraction.
10 mgm. of euglobulin. Nil.

It will be seen that a dose of 10 mgm. (1 in 7000) of either euglobulin or pseudoglobulin produced a maximal response of the muscle, after the submaximal effect of 1 mgm. of euglobulin had been obtained; but that the maximal response, to 10 mgm. of either, left the organ insensitive to the same dose of the other. Such a result is not uncommon, and seems to be especially frequent in cases, such as that quoted, in which the sensitiveness is not of a high order. It may be noted that the apparently rather smaller amplitude of contraction in response to 10 mgm. of pseudoglobulin (Fig. 2) as compared with that to 10 mgm. of euglobulin (Fig. 1), is attributable to the use of Ringer II in the former, and Ringer I in the latter case, for the purpose of comparing their suitability for this type of experiment. It is no index, therefore, of preferential sensitisation, which is excluded by the perfect mutual desensitisation.

(ii) There may be sensitisation, to either euglobulin or pseudoglobulin in higher degree than to the other. The difference was in no case very conspicuous, and its demonstration depended chiefly on the phenomena of desensitisation. Thus we might find that one horn responded maximally to 1 mgm. of euglobulin, gave a second smaller response to 10 mgm. of euglobulin, and was thereafter desensitised not only to euglobulin, but to pseudoglobulin also; while the second horn after practically similar responses to 1 mgm. and 10 mgm. of pseudoglobulin, would be completely insensitive to this protein, but still capable of giving a large response to 10 mgm. of euglobulin. In another case the reverse condition would obtain, the plain muscle desensitised to pseudoglobulin failing to respond to euglobulin, while the second horn, when desensitised to euglobulin, still responded to pseudoglobulin. A couple of examples will suffice to illustrate the point, the importance of which was that it gave us the first indication of an antigenic distinction between the two proteins, which experiments on sensitisation with the separate proteins confirmed.

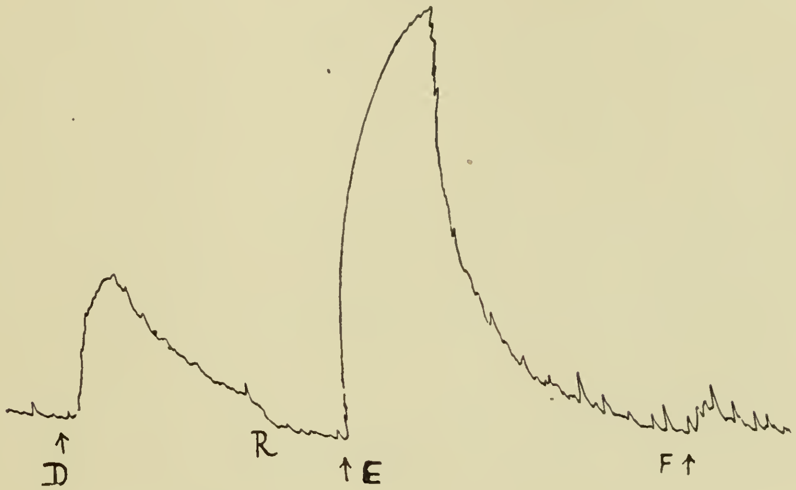

Fig. 2. From same experiment as Fig. 1. 2nd horn. D. 1 mgm. of euglobulin. E. 10 mgm. of pseudoglobulin. F. 10 mgm. of euglobulin.

Experiment 4. Guinea-pig sensitised with 0·1 cc. of horse-serum. 22nd day.

1*st horn.*

1 mgm. euglobulin. Maximal contraction.
5 mgm. euglobulin. Smaller contraction.
5 mgm. euglobulin. Nil.
5 mgm. pseudoglobulin. Nil.

2*nd horn.*

1 mgm. pseudoglobulin. Maximal contraction.
5 mgm. pseudoglobulin. Nil.
5 mgm. pseudoglobulin. Nil.
5 mgm. euglobulin. Large contraction.
5 mgm. euglobulin. Nil.

Experiment 7. Guinea-pig sensitised with 0·1 cc. horse-serum. 21st day.

1*st horn.*

1 mgm. pseudoglobulin. Good contraction.
1 mgm. pseudoglobulin. Nil.
10 mgm. pseudoglobulin. Small contraction.
10 mgm. pseudoglobulin. Nil.
10 mgm. pseudoglobulin. Nil.
10 mgm. euglobulin. Maximal contraction.

2*nd horn.*

1 mgm. euglobulin. Large contraction (? Maximal).
1 mgm. euglobulin. Nil.
10 mgm. euglobulin. Small contraction.
10 mgm. euglobulin. Nil.
10 mgm. euglobulin. Nil.
10 mgm. pseudoglobulin. Nil.
40 mgm. pseudoglobulin. Nil.

The above two records show preferential sensitisation to euglobulin. In the following the predominant sensitiveness to pseudoglobulin is shown not only in the greater effect of desensitisation to this protein on the subsequent response to euglobulin, but in the lower initial effective dose of the former.

Guinea-pig sensitised with 0·04 cc. antidiphtheritic horse-serum + 1 test dose diphtheria toxin. 24th day.

1*st horn.*

1 mgm. pseudoglobulin. Full contraction.
10 mgm. pseudoglobulin. Small contraction.
10 mgm. pseudoglobulin. Nil.
10 mgm. euglobulin. Slight contraction.

2*nd horn.*

1 mgm. euglobulin. Nil.
10 mgm. euglobulin. Good contraction.
10 mgm. euglobulin. Moderate contraction.
10 mgm. euglobulin. Small contraction.
10 mgm. euglobulin. Very small contraction.
10 mgm. pseudoglobulin. Large contraction.
10 mgm. pseudoglobulin. Nil.

In this record there is an obvious indication that complete desensitisation to euglobulin is more difficult to achieve than that to pseudoglobulin. This point is clearly discernible in a number of our records. A probably related effect is also to be seen in the above, namely, the tendency of the plain muscle to recover from the desensitisation to euglobulin, and show a revival of response to this protein at a later stage of the experiment. This may indicate a tendency of the euglobulin-antibody complex to dissociate, or it may be attributable to the physical properties of the euglobulin solution, on account of which this antigen less readily reaches and saturates the antibody in the deeper layers of the uterine muscle. We have no direct evidence in favour of either interpretation, but the fact is repeatedly illustrated in our records.

(iii) In some of our records from guinea-pigs sensitised with whole horse-serum, the distinct antigenic properties of the two globulins are made evident by the fact that, while the plain muscle responds with equal readiness to an initial dose of either eu- or pseudoglobulin, desensitisation to either of these leaves a remainder of sensitiveness to the other. This is most readily shown by producing a *relative* desensitisation, by using small doses throughout, as in the following record.

Experiment 12. Guinea-pig sensitised with 0·1 cc. horse-serum. 14th day.

1*st horn.*

1 mgm. euglobulin. Good contraction.
1 mgm. euglobulin. Small contraction.

1 mgm. euglobulin. Nil.
1 mgm. pseudoglobulin. Fair contraction.
1 mgm. pseudoglobulin. Nil.

2nd horn.
1 mgm. pseudoglobulin. Good contraction.
1 mgm. pseudoglobulin. Nil.
1 mgm. euglobulin. Fair contraction.

It is probable, to judge from the results of other experiments, that the 1st horn, when no longer giving a perceptible response to 1 mgm. of euglobulin, would have responded to 10 mgm.; and similarly with pseudoglobulin in the case of the second horn. The point to be emphasised is that, in either case, the muscle, when reduced to a condition in which it fails to respond at all to 1 mgm. of one of the globulins, still shows a definite sensitiveness to the same dose of the other.

(*b*) *Albumin.* Our earlier results seemed to support the finding of Doerr and Russ, that serum albumin did not act as an anaphylactic antigen. In a series of preparations from guinea-pigs sensitised to whole serum, we found that the plain muscle seemed completely indifferent to a dose of 5 or 10 mgm. of the albumin, subsequently giving a maximal response to 1 mgm. of either globulin. The result seemed so definite, and was obtained with such regularity in the early series, that for some time we abandoned experiments with albumin, considering its inefficacy as settled. At a later stage of the investigation six guinea pigs received the usual sensitising injection of 0·1 cc. of horse-serum, to test another point, and it happened that the uterus was tested with 1 mgm. of pure serum albumin, which produced a typical, nearly maximal, response. The uteri of the other guinea-pigs of this series were then tested with albumin, and all showed a similar high degree of sensitiveness to this protein. The difference could not be attributed to a deficiently purified albumin, since the preparation which now proved an effective stimulus was the same as that which had previously proved ineffective; and the highly purified sample obtained by recrystallising four times, as described above, was quite as effective a stimulant as that purified by the precipitation method only. The only difference between the two series was in the time elapsing between the sensitising injection and the performance of the test. In the earlier series, in which no sensitiveness to albumin was detected, the interval was two to three weeks; in the later series, in which a good reaction to albumin was observed, it was upwards of thirty days—four to five weeks. A further series of six guinea-pigs was therefore prepared, being sensitised with 0·1 cc. horse-serum and kept for twenty-nine to thirty days before testing, with the same result, that after this interval a sensitiveness of the plain muscle to albumin had developed.

There was some indication that, concurrently with the late development of the sensitiveness to albumin, the sensitiveness of the plain muscle to the globulins, which attains its maximum somewhere between the 14th and 21st days, undergoes some decline, to judge by the size of the initial dose needed to produce a pronounced reaction. The following record illustrates the point.

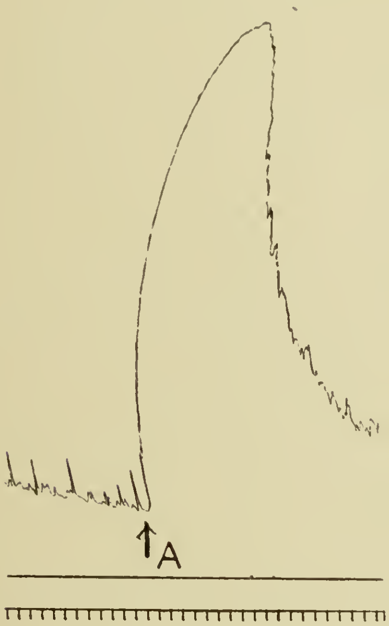

Fig. 3. Guinea-pig sensitised with 0·1 cc. of whole horse-serum. 31st day. 1st horn. A. 1 mgm. of albumin.

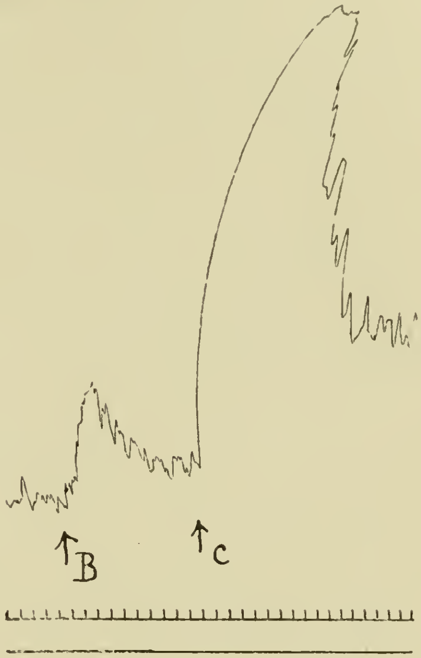

Fig. 4. Same experiment as Fig. 3. 2nd horn. B. 1 mgm. of euglobulin. C. 10 mgm. of euglobulin.

Experiment 26. Guinea-pig sensitised with 0·1 cc. horse-serum. 31st day.

1*st horn.*

1 mgm. albumin. Maximal contraction (see Fig. 3).
10 mgm. albumin. Weak contraction.
10 mgm. albumin. Nil.
1 mgm. euglobulin. Nil.
10 mgm. euglobulin. Moderate contraction.

2nd horn.

1 mgm. euglobulin. Small contraction.
10 mgm. euglobulin. Maximal contraction (see Fig. 4).
10 mgm. euglobulin. Nil.
10 mgm. albumin. Nil.

It will be noticed that though the plain muscle, in this case, showed a higher sensitiveness to albumin than to euglobulin, as judged by its response to a small dose (1 mgm.) of either, it is more readily desensitised to albumin by an effective dose of euglobulin, than to euglobulin by albumin.

The results obtained, by observing the response of isolated plain muscle, suggested that the failure of previous workers to observe an anaphylactic reaction in the serum-sensitive guinea-pig, when pure albumin was used for the reinjection, might have been due to the allowance of an insufficient incubation-period for the development of sensitiveness to this protein. It seemed desirable, therefore, to confirm, by experiments on the intact animal, the fact that true anaphylaxis to serum albumin does occur, though late in its development; in other words, to ensure that the reaction of the isolated plain muscle, *in vitro*, gives, in this case also, a genuine indication of the occurrence of the anaphylactic condition, as seen in the whole animal. A guinea-pig from the same series as that used in Experiment 26, and similarly sensitised by a hypodermic injection of 0·1 cc. of horse-serum, was therefore tested on the 32nd day after sensitisation. The albumin was made up for the reinjection in 1 % solution in physiological saline. Of this solution 1 cc. (10 mgm. of albumin) was injected into the jugular vein, exposed by a small incision under local cocaine-anaesthesia. Symptoms appeared immediately after the completion of the injection, and typical anaphylactic death followed in about 3 minutes. The post-mortem appearances were quite characteristic—fixed distension of the lungs, and retarded coagulation of the blood.

It seemed desirable to confirm, in another species of animal, this efficacy of serum-albumin as an anaphylactic antigen, provided the incubation period is sufficiently long. A dog therefore received a sensitising injection of 5 cc. of horse-serum hypodermically. On the 28th day after this injection the dog was anaesthetised by A.C.E. mixture, after a preliminary injection of morphia. Arrangements were made for recording the blood-pressure from the carotid artery, and injection into the femoral vein. The record was started, and 20 cc. of a 5 % solution in saline of the pure albumin of horse-serum were then run slowly into the vein. A profound and typical shock occurred, the blood-pressure commencing to fall rapidly during the injection, continuing to fall after the injection was completed, and remaining long at a very low level. The fall of pressure was accompanied by the typical dyspnoea, and a sample of blood, obtained while the depression persisted from the other carotid artery, showed no sign of clotting after standing for 24 hours.

Guinea-pigs sensitised to individual proteins.

(*a*) *Sensitised to euglobulin.* Sensitisation with euglobulin produced a more clearly specific sensitiveness than that with either of the other proteins. When a guinea-pig was killed 13 to 17 days after a preliminary injection of 1 mgm. of euglobulin, its uterine plain muscle responded typically to 1 mgm. or 10 mgm. of euglobulin, but was apparently indifferent either to albumin or pseudoglobulin, at any rate within the limits of dosage employed in these experiments (see Figs. 5 and 6).

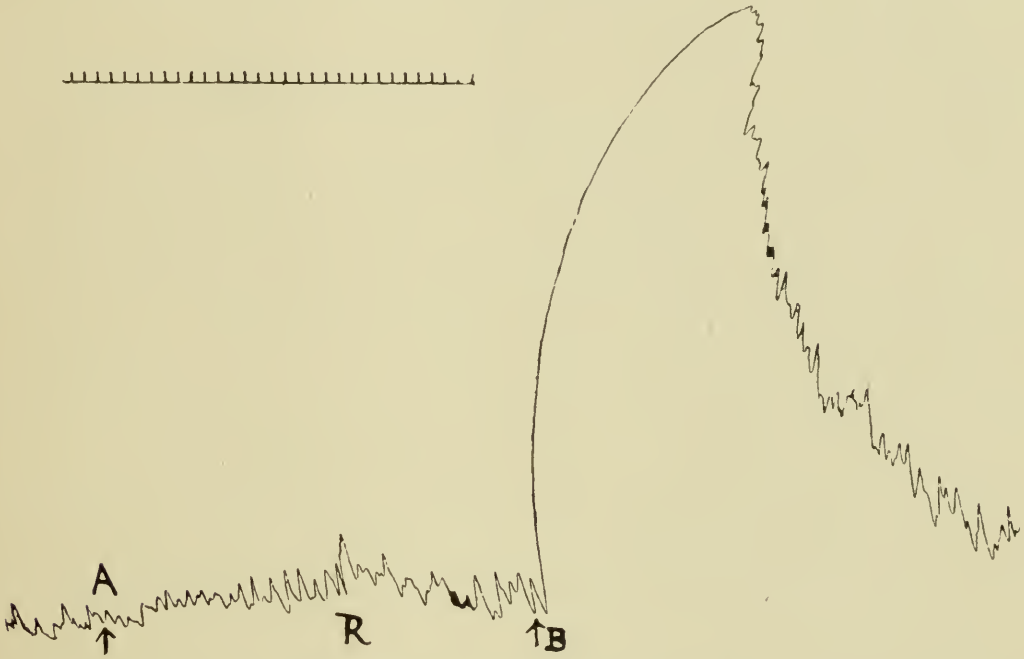

Fig. 5. Guinea-pig sensitised with 1 mgm. of euglobulin. 17th day. A. 5 mgm. of albumin. B. 1 mgm. of euglobulin.

(*b*) *Sensitised to pseudoglobulin.* The available samples of pseudoglobulin produced a sensitisation to either pseudoglobulin or euglobulin, but the sensitiveness to the former was always definitely superior, as the following record shows.

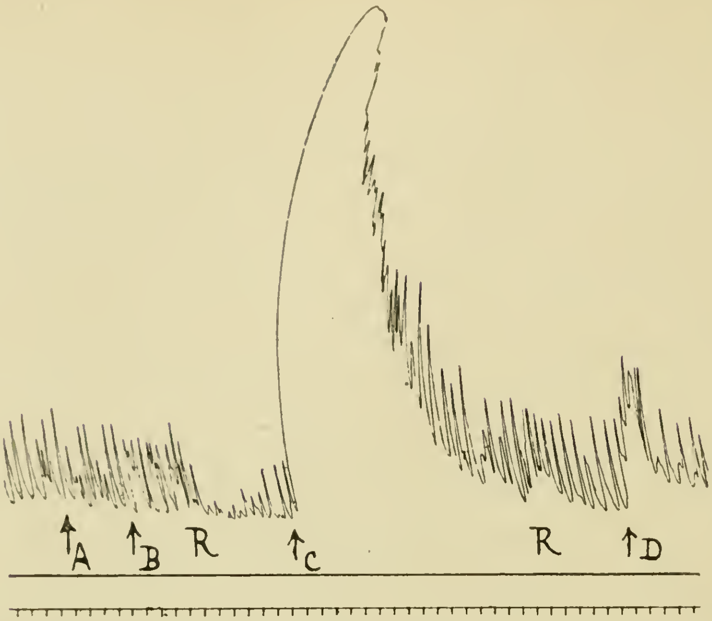

Fig. 6. Guinea-pig sensitised with 1 mgm. of euglobulin. 13th day. A. 1 mgm. of pseudoglobulin. B. 10 mgm. of pseudoglobulin. C. 10 mgm. of euglobulin. D. 10 mgm. of euglobulin.

Experiment 30. (See Figs. 7, 8 and 9.) Guinea-pig sensitised with 1 mgm. of pseudoglobulin. 22nd day.

1*st horn.*

1 mgm. pseudoglobulin. Maximal contraction.

2*nd horn.*

1 mgm. euglobulin. Moderate contraction.
10 mgm. euglobulin. Good but submaximal contraction.
10 mgm. euglobulin. Small contraction.
10 mgm. euglobulin. Nil.
10 mgm. pseudoglobulin. Good but submaximal contraction.

It is clear that 1 mgm. pseudoglobulin, as a first dose, is much more effective than 1 mgm. euglobulin under the same conditions; further that complete desensitisation to euglobulin weakens, but by no means annuls the sensitiveness to pseudoglobulin.

Experiment 35. Guinea-pig sensitised with 0·1 mgm. pseudoglobulin 18 days.

1*st horn.*

0·1 mgm. pseudoglobulin. Small contraction.
1 mgm. pseudoglobulin. Moderate contraction.
1 mgm. pseudoglobulin. Nil.
10 mgm. euglobulin. Very small contraction.

2nd horn.

1 mgm. euglobulin.	Nil.
10 mgm. euglobulin.	Maximal contraction.
10 mgm. euglobulin.	Nil.
1 mgm. pseudoglobulin.	Small contraction.

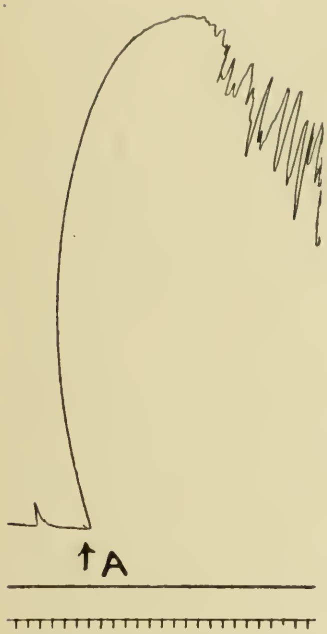

Fig. 7. Guinea-pig sensitised with 1 mgm. of pseudoglobulin. 22nd day. 1st horn. A. 1 mgm. of pseudoglobulin.

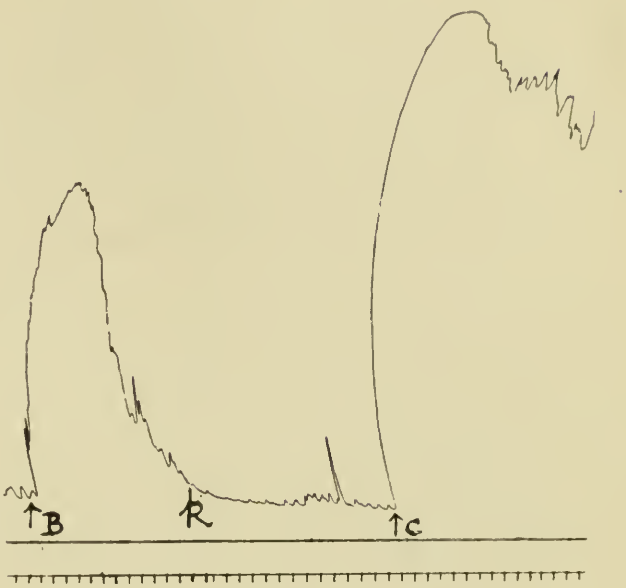

Fig. 8. Same experiment as Fig. 7. 2nd horn. B. 1 mgm. of euglobulin. C. 10 mgm. of euglobulin.

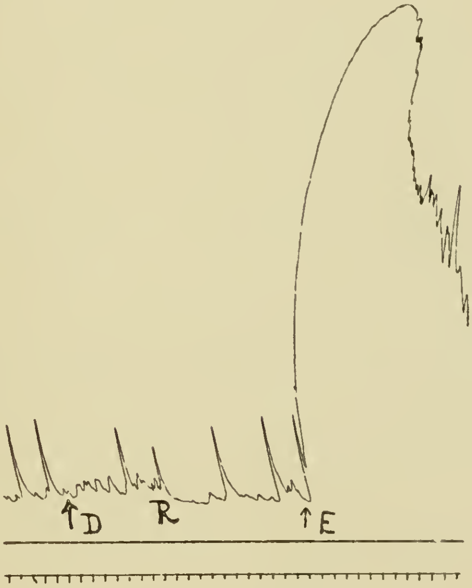

Fig. 9. Continuation of Fig. 8. D. 10 mgm. of euglobulin. E. 10 mgm. of pseudoglobulin.

In this experiment an error was made in dilution, which resulted in the dose of pseudoglobulin being throughout only one-tenth of what had been intended. The mistake, however, was not without advantage, in bringing out very clearly the preponderance of sensitiveness to pseudoglobulin. For the result showed that desensitisation to 1 mgm. of pseudoglobulin almost abolished the response to 10 mgm. of euglobulin; while after desensitisation to 10 mgm. of euglobulin, the muscle responded definitely, though not powerfully, to 1 mgm. of pseudoglobulin. It may also be noted that, whereas the first horn gave a small, but quite definite response even to 0·1 mgm. pseudoglobulin—*i.e.* to a dilution in the testing-bath of 1 in 700,000—the second horn gave no response at all to 1 mgm. (1 in 70,000) of euglobulin, but was stimulated to maximal contraction by 10 mgm. (1 in 7,000).

Sensitised to serum albumin.

All the guinea-pigs used in these tests received a sensitising hypodermic injection of 1 mgm. of purified serum albumin. Unfortunately lack of time, and the exhaustion of our small stock of purified pseudoglobulin, prevented any discriminating tests between the antigenic properties of pseudoglobulin and albumin, which would have given information of some interest. We were able, however, to show, more definitely than with the guinea-pigs sensitised to whole serum, the antigenic independence of albumin and euglobulin. Few experiments were made with isolated muscle, and it will be sufficient to quote one. It will be noted, both in this case and in the records of experiments on the intact animal, that when pure albumin is used for sensitisation, anaphylaxis to this protein, though still somewhat slowly developed, appears at an earlier stage than when the immunity reaction is complicated by the presence of the other serum proteins in the sensitising inoculum.

Experiment 35. Guinea-pig sensitised with 1 mgm. serum albumin hypodermically. 22nd day.

1st horn.

1 mgm. serum albumin. Good, but not maximal contraction.
10 mgm. serum albumin. Nil.
10 mgm. euglobulin. Nil.

2nd horn.

1 mgm. euglobulin. Nil.
10 mgm. euglobulin. Trace of response (doubtfully specific).
1 mgm. albumin. Good, but not maximal contraction.

For the experiments on anaphylaxis *in vivo* two guinea-pigs (*A* and *B*) were first sensitised, each with 1 mgm. albumin as usual. Twenty-two days later, when each weighed 250 g. *A* was tested by intravenous injection of 10 mgm. albumin in 1 cc. of saline. This caused pronounced symptoms—coughing, chattering, erection of hair, falling on the side. After about 20 minutes, however, the animal was obviously recovering, and 10 minutes later was practically normal again. Next day it was perfectly well.

On the 35th day both guinea-pigs weighed about 275 g. *B* received 5 mgm. of albumin in 0·5 cc. of saline intravenously, showed immediate pronounced symptoms, and died within 2 minutes.

A, which had survived the injection on the 22nd day, was given a second injection of 10 mgm. on the 35th day. Symptoms developed slightly more slowly than in *B*, but death occurred within 3 minutes.

Both animals showed the typical post-mortem picture of acute anaphylaxis—lungs fixed in distension, slowly clotting blood, etc.

A further series of twelve guinea-pigs (numbered I–XII) were sensitised with 1 mgm. of albumin and examined as follows.

No. of guinea-pig	Weight in g.	No. of days after sensitisation	Dose given intravenously	Result
I	275	10	Albumin 20 mgm.	Slight symptoms, recovered
II	275	10	,, 10 ,,	Nil
III	275	17	,, 20 ,,	Death in 3 mins.
IV	250	17	,, 10 ,,	,, 4 ,,
V	250	17	,, 5 ,,	,, 5 ,,
VI	225	17	,, 2 ,,	,, 3 ,,
VII	215	17	,, 1 ,,	,, 3 ,,
X	220	27	Euglobulin 5 mgm.	Nil
XII	210	27	Albumin 0·5 mgm.	Death in 2 mins.
X (2nd injection)	220	28	,, 1 mgm.	,, 4 ,,
I (2nd injection)	300	30	Albumin 10 mgm.	Nil
II (2nd injection)	300	30	,, 1 ,,	Nil

Guinea-pig VIII was used for the isolated muscle test quoted above; guinea-pigs IX and XI died from accidental causes before testing.

There are several points worth noting in the above table. The sensitiveness to albumin is practically non-existent on the 10th day, by which sensitiveness to whole serum is well developed in animals previously injected with this. By the 17th day, however, in this series a very high sensitiveness to albumin has appeared, the lower limit of fatal dosage for guinea-pigs of this size not being reached in the experiment. The experiments on the 27th and 28th days show that there is no reaction to euglobulin in these guinea-pigs, even in a dose at least ten times as large as the fatal dose of albumin; nor does the injection of euglobulin notably modify the effect of albumin given on the next day. Lastly, it is seen that, when large injections have been given on the 10th day, *i.e.* before sensitiveness to albumin has appeared, no sensitiveness of the animal to albumin can be detected by intravenous injections given on the 30th day. It has been shown by one of us that such lack of apparent sensitiveness may be due either to genuine desensitisation of the tissues, or to an excess of antibody in the circulating fluids, protecting from the antigen tissues which, when isolated, are highly sensitive. It must remain an open question, for the present, which of these two conditions accounted for the lack of intoxication in this instance.

Note on Anaphylaxis to Pure Egg-albumins.

The production in the guinea-pig of a high degree of anaphylaxis to the pure, crystallised albumin of the hen's egg has been previously described by Armit [1910] and by Wells [1908]. Wells [1911] has also shown that ovovitellin, from egg-yolk, acted as a quite separate antigen from the proteins of egg-white[1]. In the preceding sections we have given evidence of the separate action, as anaphylactic antigens, of the different proteins of one serum. By way of contrast it seems appropriate to make preliminary mention here of some experiments made by one of us (H. H. D.) on anaphylaxis to the pure crystalline albumins from the egg-white of hens and ducks, in the preparation of which Dr A. J. Ewins kindly co-operated. The albumins were crystallised by the familiar method of Hopkins [1899], three successive crystallisations being given to each sample. The eggs were obtained from a private source, and there can be no question of any mistake in specific identification. The albumin from the ducks' eggs was rather more difficult to crystallise than that from hens, a larger addition of acid being required to initiate the first crystallisation. When once obtained in clean crystals, however, it was recrystallised as readily as that of the hen. The final crop of crystals, in both cases, was dialysed free from ammonium sulphate, and the watery solutions were evaporated to dry scale-preparations at room-temperature *in vacuo*, solutions being then prepared as required. Female guinea-pigs received injections of 0·1–1 mgm. of either albumin, and the isolated uterine plain muscle was tested for sensitiveness after three weeks. The result can be stated in a line. There was no evidence of any antigenic distinction between the two albumins. The plain muscle of guinea-pigs, which had been injected with hen's albumin was quite as sensitive to duck's albumin as to hen's, and the same was true of preparations from guinea-pigs injected with duck's albumin. Moreover an effective dose of either albumin was followed by complete insensitiveness to the other, irrespective of which had been used as the sensitising dose. If the notebook record had been lost, it would have been impossible, from the response of the plain muscle, to form any opinion as to which albumin had been used for sensitisation. Fig. 10 illustrates the corresponding reactions of the two uterine horns from a guinea-pig sensitised with hen's albumin. It may be added that neither preparation had any effect on uterine muscle from normal, unsensitised guinea-pigs.

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[1] There is a somewhat extensive literature on the crossed specificity of different egg-whites, which has not been dealt with here, since the experiments made by us at present have been concerned solely with the crystalline albumins.

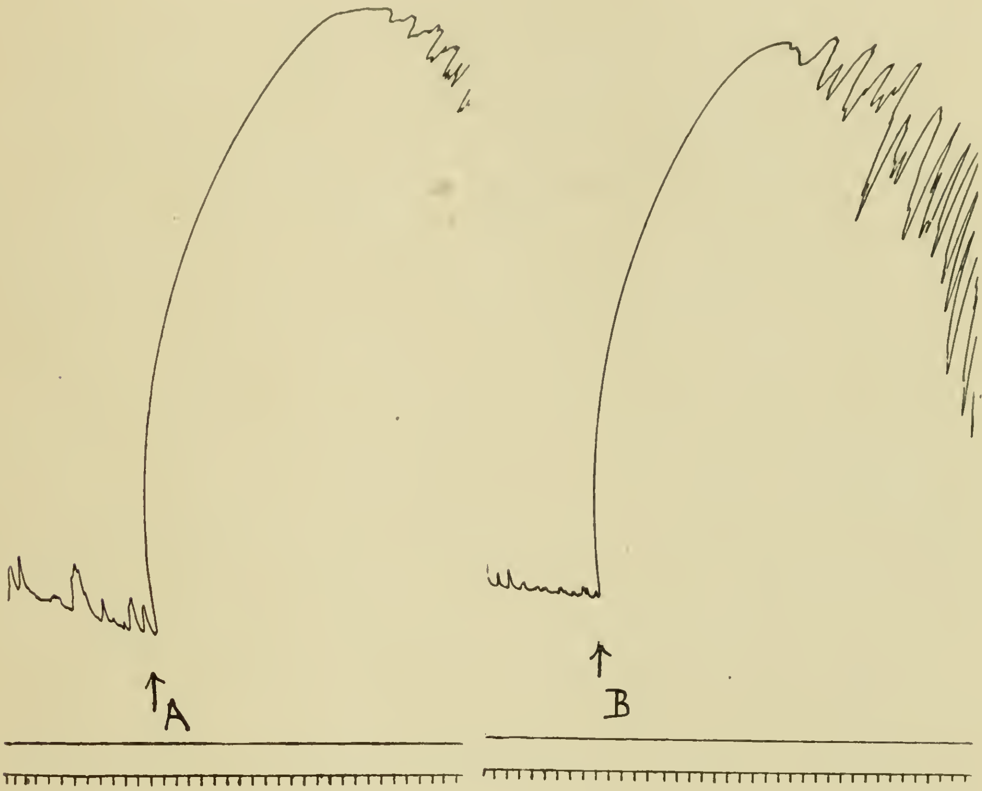

Fig. 10. Guinea-pig sensitised with 1 mgm. of crystalline albumin of hen's egg-white. 22nd day. A. 1st horn. Effect of 0·05 mgm. duck's egg-albumin. B. 2nd horn. Effect of 0·05 mgm. hen's egg-albumin.

SUMMARY AND DISCUSSION.

The following are the main conclusions to be drawn from the above experiments:

1. Each of the three proteins separable from horse-serum by their physical and chemical properties—euglobulin, pseudoglobulin, albumin—can act as an anaphylactic antigen.

2. A guinea-pig which has received a sensitising injection of one of these proteins is more sensitive to that one than to either of the others from the same serum. In some cases the sensitisation seems to be rigidly "specific," as when euglobulin produces sensitiveness to euglobulin but not to pseudoglobulin, or when albumin produces a high degree of sensitiveness to albumin but none to euglobulin.

3. The sensitiveness of the guinea-pig to albumin is later in development than that to the globulins; this difference possibly accounts for the fact that previous authors failed to detect the sensitising property of albumin. The difference is especially marked when sensitisation is carried out with whole serum, containing all three.

4. An effective dose of any of the proteins, to which the guinea-pig's plain muscle has been sensitised, partially or completely desensitises it to the other proteins of the same serum.

5. The crystalline albumins from the white of the eggs of hens and ducks show no distinction of antigenic properties.

The effects of "crossed" desensitisation, in particular, may give the impression that there is a considerable community of antigenic function among the proteins of one serum. Such an impression, however, is not completely justified, since one of us (H. H. D.) has previously shown that, when a guinea-pig is simultaneously sensitised to two or more antigens of widely different origin, and of undoubted antigenic independence—*e.g.* egg-white and horse-serum—desensitisation of the plain muscle to one may greatly weaken the sensitiveness to the other. On the other hand, the fact that sensitisation with one of the globulins frequently produces a smaller, but quite definite sensitiveness to the other, shows that, at any rate, the preparations of these two substances at our disposal were not completely independent in their antigenic properties. But the question necessarily arises whether this overlap is a genuine phenomenon, or is due to the fact that, even in such elaborately purified preparations as those which we used, the separation was not ideally perfect. This possibility is worthy of consideration, in that the amount of a protein needed to evoke some degree of sensitiveness is almost inconceivably small. Wells [1908], for example, succeeded in sensitising guinea-pigs with 1/20,000 milligram of egg-albumin. This explanation of the overlap in sensitisation is favoured by the evidence that it appears most prominently when the impurity of the sensitising protein is most likely to be considerable. The purification of euglobulin is facilitated by its insolubility in weakly acidulated water. We have found that a rigidly specific sensitiveness to euglobulin occurs comparatively frequently. On the other hand, we never succeeded, by injection of pseudoglobulin in producing a sensitiveness to pseudoglobulin alone; sensitiveness to euglobulin in addition was always detectable, though it might be relatively weak, especially when the sensitising dose was small. But the purification of pseudoglobulin from the last traces of euglobulin is so difficult, that it may be doubted whether it is possible by existing methods. It is similarly difficult to make a sharp separation between pseudoglobulin and albumin, and we think it probable that a similar overlap of sensitisation would have been detected between these two proteins. Unfortunately the material was exhausted before this point had been put to the test. Again, if we regard the overlap of antigenic properties as due to incomplete separation, it would be expected that the sharpest distinction would be observed between euglobulin and albumin, each of which can be obtained with tolerable certainty uncontaminated by the other. The results were quite in accordance with this expectation, since the plain muscle from a guinea-pig sensitised with euglobulin seemed quite indifferent to albumin, and *vice versâ*. On the whole, then, the evidence seems to us in favour of the view that the different proteins of a serum, in so far as they can be obtained pure from one another, act as independent anaphylactic antigens.

This narrowing down of immunological "specificity," to a discrimination between the different pure proteins of a single tissue of one species, is by no means new. Wells [1911], as above mentioned, found evidence of complete antigenic independence between the "ovovitellin" of egg-yolk, on the one hand, and the proteins of egg-white on the other; he found, however, a considerable overlap in the antigenic properties of the albumin and globulin of egg-white, however carefully they were purified. Ovomucoid was again an independent antigen. Wells and Osborne [1911, 1913] have furnished numerous instances in which the different pure proteins from one plant species act as separate antigens, while the corresponding pure proteins from allied species have common antigenic properties. Such evidence gives to the fundamental problem of immunity a more hopeful aspect, by its suggestion that the antigenic distinction is possibly dependent on such properties of the different proteins as may be open to the ordinary methods of chemical and physical enquiry, and not necessarily on some biological difference of so mysterious a nature, as to elude investigation by such methods. Our own evidence of the antigenic independence of the proteins from one blood-serum, and of the community of antigenic function between the pure albumins of egg-white from species so widely separated as the fowl and the duck, has the same tendency. It seems clear to us that every sensitisation with a whole serum, for example, is in reality a complex multisensitisation; and that it is essential to work with individual pure proteins, in any attempt to investigate the nature of the difference, of which antigenic independence is an expression.

The question has often been debated whether the difference is a chemical or a physical one, without giving these terms any greater precision of application. One possibility of chemical difference we are in a position to eliminate. An analysis of the separated proteins from horse-serum, used by us in these experiments, had been carried out by one of us [P. Hartley, 1914] by the aid of van Slyke's method. It was found that albumin, as others had shown, differed from the globulins in yielding, on hydrolysis, a higher proportion of the diamino-acids, and in containing a higher proportion of cystine. It was further shown that, of the diamino-acids, the excess yielded by albumin, as compared with the globulins, was greatest in the case of lysine, which albumin contained in about a 50 % higher proportion. No such difference was found between the two globulins, which, as regards the distribution of their nitrogen among the different amino-acids, showed no difference of any note. There is no help to be obtained, therefore, in this direction towards an explanation of the antigenic difference between the globulins. It is clear, however, that there is abundant room for difference of chemical structure even between protein molecules built up from the same amino-acids in identical proportions. Indeed, if the highly specific combining properties, which give a protein molecule its antigenic character, are related to chemical structure at all, it seems likely that they would be as much dependent on the pattern of the structure of the molecule, on the order in which the amino-acids are linked up in it, as on their nature and the proportions in which they are present. A relation between the stereo-chemical and antigenic characters of the protein molecule is, indeed, definitely suggested by the observation of Ten Broek [1914], who found that a protein racemised by Kossel's method had lost all power of acting as an antigen, just as Dakin and Dudley [1913] found that it had lost its susceptibility to the action of proteolytic enzymes. Dakin [1912] has given reason for believing that the amino-acids which escape racemisation in this process, and are therefore obtained in the natural, optically active condition on subsequent acid hydrolysis of the racemised protein, are those occupying the terminal positions of the peptide chains out of which the protein molecule is built. Dudley and Woodman [1915], using Dakin's method, found evidence of a structural difference of the kind under discussion between the caseinogens of cow's and of sheep's milk—proteins consisting of identical amino-acids in identical proportions. In the case of sheep's caseinogen the tyrosine wholly and the lysine largely escaped racemisation, while they were both completely racemised in that of cow's caseinogen. The instance is an unsatisfactory one for our present purpose, since the antigenic properties of caseinogen are relatively feeble, and the caseinogens from different species show no clear disparity of antigenic properties. In the only other case of proteins with identical amino-acid content which have hitherto been thus compared, the euglobulin and pseudoglobulin of colostrum, there is presumptive evidence of an antigenic difference of the same kind as that which we find existing between the corresponding serum-proteins. In this instance Dudley and Woodman detected no difference by the racemisation method. So that, so far as evidence of this kind has been obtained at present, it fails to support, and might even seem to discredit the view that antigenic properties depend on the structural pattern of the protein molecule. We have an example of structural difference, without antigenic disparity, and another, with no structural difference detected, in which some degree of antigenic independence may be presumed. It must be remembered, however, that the method reveals only one kind of structural difference, the effect of which on the antigenic properties might well be subsidiary to other differences, which the method fails to detect. The observation that two similar proteins from different species, the caseinogens of the cow and sheep, while as yet indistinguishable by other methods, show a definite difference in the order of amino-acid linkage, remains highly significant, and seems to indicate the lines along which the possibility of a connexion between antigenic properties and chemical structure may yet be investigated.

It has not been assumed, in thus considering the possibility of a chemical basis for antigenic specificity, that the combination between antigen and antibody is of the type of an ordinary chemical union. There is, of course, much evidence indicating that the association has the character rather of an adsorption. This, however, by no means precludes the possibility that it may be conditioned ultimately by the structure of the antigenic molecule. There is similarly strong evidence in favour of regarding the association between an enzyme and its substrate as an adsorption; yet it is essentially dependent on the configuration of the substrate molecule. Again, Barger and his co-workers [1915] have demonstrated the connexion between the formation of coloured adsorption-compounds with iodine and a certain type of structure in organic compounds. They also showed, however, the dependence of the reaction on surface-phenomena, in that a colloidal solution, or suspension of the substance in amorphous aggregates, was necessary for the formation of the typical coloured complex with iodine. It is not improbable that the antigenic behaviour of a protein is similarly conditioned by its state of dispersion. Miss Chick [1914] and others have given reason for considering euglobulin as possibly formed by association of pseudoglobulin with a lipoid, which gives to euglobulin its phosphorus content and its characteristic physical properties. It is conceivable that the antigenic difference between a pseudoglobulin and the euglobulin from the same serum may be due to the peculiar physical characteristics conferred on the euglobulin by associated lipoid. We hope later to test this possibility by comparing the antigenic action of a pseudoglobulin, with that of an artificial "euglobulin" made from it. But even if that were the case, such characteristics could hardly be more than one factor in the phenomenon of specificity; and to form an idea of its significance, it would be necessary to examine the degree of antigenic independence exhibited by pure euglobulins from the sera of different species. Using the purest proteins obtainable as antigenic units, it should eventually be possible to compare different proteins from the same species, and similar proteins from different species, in respect of their molecular configuration and also of their physical properties, and to trace the relation of each type of character to the antigenic individuality of the substances examined.

A word may be added on some practical bearings of our results. Since all the proteins of a serum can act as anaphylactic antigens, it is evident that the ideal to be aimed at, in concentrating the curative element in a specific immune serum, is simply the reduction of the ratio of total protein to antitoxic value; for the purpose of reducing serum-reactions, the elimination of albumin seems to be as important as that of euglobulin, when the pseudoglobulin is the fraction carrying the therapeutic power. Our observation of the relatively long latent period of the sensitiveness to albumin is also suggestive in connexion with the successive crops of serum rash, which have been recorded in certain patients as the result of one injection of a serum. It has been suggested [Goodall, 1907] that these represent separate reactions to the sera of the different horses which may have contributed to the serum. It seems to us more reasonable to suppose that they represent the successive appearances, at different time intervals, of sensitiveness to the different serum-proteins.

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Nº 10

VIII. THE CONDITIONS OF ACTIVATION OF WASHED ZYMIN AND THE SPECIFIC FUNCTION OF CERTAIN CATIONS IN ALCOHOLIC FERMENTATION.

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It was observed by Oppenheimer [1915] that the addition of a small amount of a pyruvate (1 per mille) or of pyruvic acid (1 %) to a mixture of Lebedev's maceration juice (prepared from dried yeast) and glucose caused an increase in the rate of fermentation of the glucose, and that a similar, but much smaller, acceleration was produced by the addition of acetaldehyde (1 : 200,000). Neuberg [1915, p. 75] somewhat later made a similar observation and extended it to the fermentation of fructose, mannose, cane sugar and maltose and also found [1915, p. 83] that other α-ketonic acids produced an analogous effect. He further attempted [Neuberg and Schwenk, 1915] to activate yeast-juice, which had been freed from coenzyme by dialysis, and zymin (acetone-yeast), rendered inactive by washing, by adding to them salts of individual α-ketonic acids and a phosphate. In this, however, he was unsuccessful, but by adding a mixture of salts of a large number of α-ketonic acids (pyruvic, α-ketobutyric, α-ketoisovaleric, α-ketocapronic, phenylglyoxylic, phenylpyruvic, *p*-hydroxyphenylpyruvic, hydroxypyruvic, oxalacetic and α-ketoglutaric) along with dipotassium hydrogen phosphate, he succeeded in obtaining a small amount of fermentation (of the order of 5 cc. of CO_2 from excess of sugar by the action of 20 cc. of inactivated maceration juice or 2 g. of inactivated zymin), and concluded that this mixture could in part replace the coenzyme.

On repeating these experiments it was found that zymin, which had been prepared from top-yeast and rendered inactive by thorough washing, was readily activated by potassium pyruvate in presence of a suitable concentration of a phosphate. The experiments were then extended to acetaldehyde, since this substance is the immediate product of the decomposition of salts of pyruvic acid by the carboxylase of the zymin, which, as has been previously shown [Harden, 1913; Neuberg and Rosenthal, 1913] is not inactivated by washing. The first experiments were carried out in the presence of sodium phosphate and were unsuccessful, but it was subsequently found that, when the sodium phosphate was replaced by the potassium or ammonium salt, acetaldehyde activated the zymin in the same manner as the pyruvate.

It thus appears that potassium and ammonium ions play some special part in the process of fermentation in which they cannot be replaced by sodium ions. The presence of sodium ions is not in itself inhibitory, since activation occurs in their presence, provided only that potassium or ammonium ions are also present. This observation complements in an interesting manner the discovery by Adolf Mayer [1874] that potassium phosphate cannot be replaced by the sodium salt in a synthetic culture medium for yeast.

The fact that washed zymin can be activated by acetaldehyde in presence of a phosphate is of considerable interest from several points of view. In the first place it is consistent with, and may even be regarded as strong evidence in favour of, the theory now held by many investigators [for literature see Harden, 1914] that acetaldehyde is an intermediate product in alcoholic fermentation and is reduced in that process to alcohol by hydrogen liberated at a previous stage of the decomposition. According to this view the acetaldehyde would fulfil the function of an activator by serving as an acceptor for the hydrogen and would thus enable the reaction to start. It is possible however that other reducible substances would act in the same way and this would to some extent lessen the weight of the evidence in support of the acetaldehyde theory although it would be in agreement with the view that a hydrogen acceptor is a factor necessary for fermentation. This question will form the subject of further experiments as soon as circumstances permit.

These considerations lead directly to the question whether acetaldehyde can be regarded as constituting the coenzyme of yeast-juice. There is much in favour of this idea, but in view of the possibility that some other reducible substance may play the same part as acetaldehyde it is impossible to come to a definite conclusion without further investigation respecting the actual presence of acetaldehyde in yeast-juice and the activation of yeast-juice freed from coenzyme by dialysis, ultrafiltration and continued fermentation. As regards washed zymin, acetaldehyde certainly acts in all respects as a coenzyme, provided that phosphate and potassium or ammonium ions are present in suitable concentrations.

A second consideration is that the possibility of reactivating washed zymin by means of acetaldehyde and a phosphate affords a means of examining the effect of various cations and anions on the initiation and course of the fermentation and this opens a field of enquiry which may be expected to yield interesting results.

The stimulating effect of pyruvate and acetaldehyde on fermentation by yeast-juice observed by Oppenheimer and by Neuberg, also becomes of great interest in the light of these results and will form the subject of a further communication.

Experimental.

The zymin used in these experiments was prepared from a top-fermentation yeast employed in an English brewery by the usual method of treatment with acetone. It was washed five times with 6 parts of water in the manner previously described [Harden and Young, 1911] each washing lasting $\frac{3}{4}$ hour, and was then made up to a given volume with water. The same sample of zymin was used throughout the experiments quoted so that the results of experiments done with different batches are roughly comparable. All the fermentations were carried out in presence of toluene at 25°, the solutions being previously saturated with CO_2 at this temperature and incubated for 10—15 minutes before readings were made. The carbon dioxide evolved was collected and measured in the apparatus previously described [Harden, Thompson and Young, 1910] and the figures given are cc. of gas at atmospheric pressure and temperature.

Experiments with zymin.

I. The activation of washed zymin by potassium pyruvate and potassium phosphate and the effect of the replacement of these salts by the corresponding sodium salts.

14 g. of zymin were washed and made up to 80 cc.

In each experiment 10 cc. of this suspension (1·75 g. zymin) were used along with 1 g. of glucose and water to a total volume of 20·6 cc. To five quantities were added:

Exp. 1. 2 cc. 0·5 M K_2HPO_4.
,, 2. 2 cc. 0·5 M K_2HPO_4 + 3 cc. 1 % pyruvic acid (as K salt).
,, 3. 3·3 cc. 0·3 M Na_2HPO_4 + 3 cc. 1 % pyruvic acid (as Na salt).
,, 4. 1·6 cc. 0·3 M Na_2HPO_4 + 3 cc. 1 % pyruvic acid (as Na salt).
,, 5. 10 cc. boiled washings.

Time		Evolution of CO_2 in cc.				
h.	m.	1	2	3	4	5
1	5	0·5	12·6	1·9	2·1	11·1
2	5	0·5	20·4	2·1	2·8	17·6
20	20	2·9	55·8	2·5	6·6	69

In presence of the sodium salts (Nos. 3 and 4) little more CO_2 was evolved than in the control (No. 1) in the absence of coenzyme, whereas in the presence of the potassium salts (No. 2) the fermentation is almost as vigorous as in the presence of boiled washings (No. 5).

II. The activation of zymin by acetaldehyde and potassium phosphate or ammonium phosphate and the effect of the replacement of these salts by sodium phosphate.

A. *Exp.* 6. This was carried out along with the foregoing series. To the mixture of zymin glucose and toluene were added 1 cc. acetaldehyde solution (0·7 %) + 2 cc. 0·5 M K_2HPO_4.

Time		Evolution of CO_2 in cc.	
		Control	
h.	m.	No. 1 (above)	6
1	5	0·5	13·3
2	5	0·5	19·8
20	20	2·9	52·4

B. 10 cc. of washed zymin suspension and water to 20·6 cc. To six quantities were added:

Exp. 7. 10 cc. boiled washings + 1 g. glucose.
,, 8. 3 cc. 0·5 M K_2HPO_4 + 1 g. glucose.
,, 9. 3 cc. 0·5 M K_2HPO_4.
,, 10. 3 cc. 0·5 M K_2HPO_4 + 1 g. glucose + 1 cc. acetaldehyde.
,, 11. 2 cc. 0·5 M K_2HPO_4 + 1 g. glucose + 1 cc. acetaldehyde.
,, 12. 1·6 cc. 0·3 M Na_2HPO_4 + 1 g. glucose + 1 cc. acetaldehyde.

Time		Evolution of CO_2 in cc.					
h.	m.	7	8	9	10	11	12
	55	14·1	0·2	0·5	14·2	11·7	0·9
1	55	25·1	0·6	1·1	29·6	19·5	1·6
3	0	33·2	0·7	1·3	37·7	23·6	2·3
18	10	89	0·8	1·3	80·1	51·6	2·3

The amount of sodium phosphate added was only half the equivalent of the K salt because a previous experiment had shown that even in the presence of a small concentration of K ions, no fermentation occurred in presence of the full equivalent.

C. 10 g. zymin to 60 cc. 10 cc. of zymin suspension + 1 g. glucose were taken. Total vol. = 20·6 cc. To three lots were added:

Exp. 13. 10 cc. boiled washings.
,, 14. 2 cc. 0·5 M K_2HPO_4.
,, 15. 2 cc. 0·5 M $(NH_4)_2HPO_4$ + 1 cc. acetaldehyde.

Time		Evolution of CO_2 in cc.		
h.	m.	13	14	15
1	10	8·1	1·4	6·5
3	25	16·1	1·5	15·6
20	35	39·8	1·5	34·6

Here again acetaldehyde and potassium phosphate (Nos. 10, 11) or ammonium phosphate (No. 15) produce a good fermentation whereas acetaldehyde and sodium phosphate (No. 12) are without action.

III. Negative effect of potassium ions and acetaldehyde in absence of mineral phosphate.

Six quantities of 10 cc. of zymin suspension + 1 g. glucose + water to 20·6 cc. were taken and to these were added:

Exp. 16. 10 cc. boiled washings.
,, 17. 3 cc. 0·5 M K_2HPO_4 + 1 cc. acetaldehyde.
,, 18. 3 cc. M $KHCO_3$ + 1 cc. acetaldehyde.
,, 19. 3 cc. M $NaHCO_3$ + 1 cc. acetaldehyde.
,, 20. 1·6 cc. 0·3 M Na_2HPO_4 + 1 cc. acetaldehyde.
,, 21. 0·8 cc. 0·3 M Na_2HPO_4 + 1 cc. acetaldehyde.

Time		Evolution of CO_2 in cc.					
h.	m.	16	17	18	19	20	21
1	10	11·7	14·4	2·1	1·3	0·8	1·5
2	10	18	28·3	2·3	1·7	1·4	1·9
18	10	69	74·9	3·3	3·8	1·5	2

This shows that K ions and acetaldehyde in the absence of phosphate (No. 18) are incapable of producing fermentation and further that the same is true of Na ions (No. 19) and that the reduction of the concentration of sodium phosphate does not render it efficacious as an activator (No. 21).

Experiments with dried yeast.

Since dried yeast can be inactivated by washing [Euler and Bäckström, 1912] it can readily be used for experiments on activation. It is however not so satisfactory as zymin since, probably owing to the presence of a certain proportion of living cells, some samples yield irregular results. The yeast was dried in the air at 37° and then ground in a coffee mill. It was washed in a similar manner to the zymin.

IV. Effect of the pyruvates and phosphates of potassium and sodium.

20 g. yeast, washed four times and made to 100. 15 cc. yeast suspension + 1 g. glucose + 1 cc. toluene; water to 30·6 cc.; five quantities were taken.

Exp. 22. 5 cc. 1 % pyruvic (as K salt).
,, 23. 5 cc. 1 % ,, ,, + 1·5 cc. 0·5 M K_2HPO_4.
,, 24. 5 cc. 1 % ,, ,, + 2·5 cc. 0·3 M Na_2HPO_4.
,, 25. 5 cc. 1 % ,, (as Na salt) + 1·5 cc. 0·5 M K_2HPO_4.
,, 26. 5 cc. 1 % ,, ,, + 2·5 cc. 0·3 M Na_2HPO_4.

Time		Evolution of CO_2 in cc.				
h.	m.	22	23	24	25	26
	20	2·6	9·7	15·6	13·1	1·4
1	10	5·6	16·2	22·8	20·2	2·7
2	5	6·7	24·3	—	—	3·9
3	5	7·1	34·1	—	—	5·2
19	20	9·9	71·1	—	—	8·6

Here the substitution of sodium for potassium salts (No. 26), in equivalent amounts has resulted in the complete absence of any evolution of CO_2 beyond that in the control in the absence of phosphate (No. 22). That the presence of sodium ions is not seriously harmful is shown by a comparison of Nos. 24 and 25 with No. 23.

V. Effect of substituting sodium phosphate for potassium phosphate in presence of acetaldehyde.

The dried yeast was treated as in IV. 15 cc. yeast suspension + 1 g. glucose + 1 cc. toluene; water to 30·6 cc.

Exp. 27. 1·5 cc. 0·5 M K_2HPO_4.
,, 28. 1·5 cc. 0·5 M K_2HPO_4 + 1·4 cc. 1 % acetaldehyde.
,, 29. 2·5 cc. 0·3 M Na_2HPO_4 + 1·4 cc. 1 % acetaldehyde.

Time		Evolution of CO_2 in cc.		
h.	m.	27	28	29
1		1·5	25·5	2·9
3		1·5	41·8	3·2
5	30	1·7	58·3	3·8
21	45	4·4	86·3	6·1

The contrast here between the action of equivalent quantities of sodium and potassium phosphate is very marked.

VI. Effect of the addition of various chlorides in presence of sodium phosphate and acetaldehyde.

Dried yeast, treated as above. 15 cc. of yeast suspension + 1 g. glucose + 1 cc. toluene + 0·5 cc. 0·3 M Na_2HPO_4 + 5 cc. 0·1 % acetaldehyde; water to 30·6 cc. Four quantities.

Exp. 30. 1·5 cc. M KCl.
,, 31. 1·5 cc. M NH_4Cl.
,, 32. 1·5 cc. M NaCl.
,, 33. Control.

Time		Evolution of CO_2 in cc.			
h.	m.	30	31	32	33
1	15	7·7	7·8	0·3	1·9
2	15	10·6	11·1	2·5	2·8
18		11·6	13·4	3·3	3·3

This shows that whereas the addition of KCl (No. 30) or NH_4Cl (No. 31) to a mixture containing inactivated dried yeast, acetaldehyde and sodium phosphate causes a definite, though small, degree of fermentation, that of NaCl (No. 32) has no effect, the amount of CO_2 evolved being the same as in the control (No. 33).

SUMMARY.

1. In the presence of potassium phosphate, zymin and dried yeast, which have been inactivated by washing, can be activated by the addition of a pyruvate or acetaldehyde.

2. A specific difference in relation to alcoholic fermentation exists between the ions of sodium on the one hand and of potassium and ammonium on the other.

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N° 11

THE EXCYSTATION OF ENTAMOEBA HISTOLYTICA (TETRAGENA) AS AN INDICATION OF THE VITALITY OF THE CYSTS.

BY

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IN the course of an experimental investigation undertaken to ascertain the value of emetine as a prophylactic for amoebic dysentery we have found kittens extremely refractory to the cysts of *E. histolytica* (*tetragena*)—that is, only a very small proportion (one out of twelve, after a three weeks' incubation period) of those fed with faeces containing large numbers of cysts become infected. We were thus led to endeavour to cultivate the Entamoeba from the cysts in order to obtain, if possible, stock cultures of active forms for rectal inoculation. Owing to the fact that we are going abroad very shortly, this work has now to be broken off for the time being. As we have found a method of causing the cysts present in the faeces to excyst and liberate the contained Entamoeba, we think it worth while to indicate briefly the results obtained, since this method furnishes the most reliable, and, indeed, the only conclusive, evidence of the vitality of the cysts. This is a point of the utmost practical importance as bearing upon the duration of life and the infective power of the cysts under varying environmental conditions.

A sample of faeces containing a good infection with cysts is well emulsified with saline (or water) and filtered through three or four layers of fine gauze, to remove the larger faecal particles. The filtrate is then centrifugalized and the deposit washed three or four times, the supernatant liquid, which contains large numbers of bacteria but no cysts, being pipetted off each time. By this means toxic products are removed and the cysts are concentrated in a mass of fine faecal débris. As excysting agents we

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have tried pepsin, in an acid medium, bile, and pancreatic extract, either alone, consecutively, or together, as appeared indicated, but the only success we have had has been with pancreatic extract used alone. The preparation used is Benger's *liquor pancreaticus.* A mixture is made up in the following proportions: Nutrient broth 5 parts, liquor pancreaticus 2 (or 3), cyst-containing sediment 1 part. This mixture is then incubated for five to seven hours at 37° C. At the same time a drop of the mixture is placed on a slide, covered, and the cover-slip ringed to prevent evaporation; this is also incubated. This observation-preparation serves as a control to the tube and can be taken out and rapidly examined whenever desired, to ascertain how excystation is progressing; after five or six hours excystation is usually taking place.

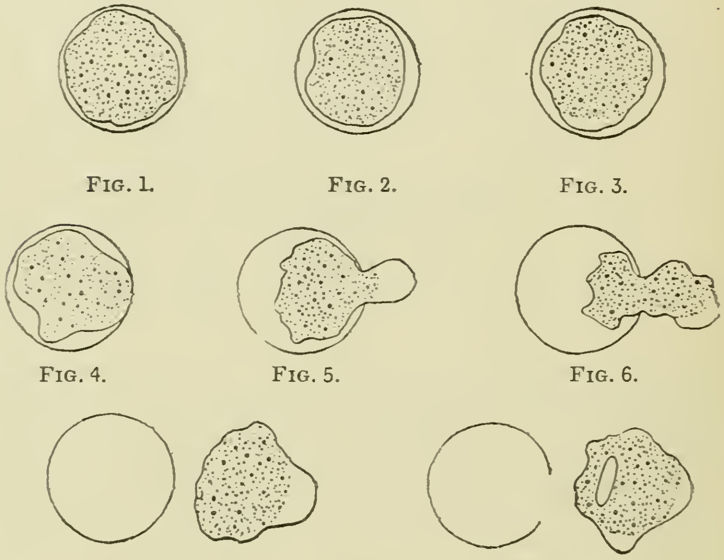

FIG. 1. FIG. 2. FIG. 3.

FIG. 4. FIG. 5. FIG. 6.

FIG. 7. FIG. 8.

Excystation of *Entamoeba histolytica* (*tetragena*); from sketches made at the time of observation. Figs. 1, 2, and 3, Slight amoeboid changes within the cyst. Fig. 3 shows a slight development of ectoplasm (this is unusual, and only seen if excystation is apparently delayed). Figs. 5 and 6, Emergence of the Entamoeba through an aperture in the cyst membrane. Figs. 7 and 8, Two liberated Entamoebae, with their empty cysts (seen in optical section); in the latter figure the aperture in the membrane is in focus, and this Entamoeba possesses a "chromidial block" (the endoplasm is dotted to indicate its finely granular character; the ectoplasm is left clear).

Even of cysts in freshly passed faeces, the majority of which appear quite normal, only a small proportion excyst. The first indication of approaching excystation is that the protoplasm becomes very slightly retracted from the cyst-membrane and appears somewhat crinkled (Fig. 1). By careful watching it can be seen that the contour of the body is very slowly changing its shape, that is to say, the Entamoeba is becoming slightly amoeboid (Figs. 2, 3). The nuclei are not visible; it may be pointed out that when the nuclear rings are clearly visible in a fresh *tetragena* cyst, it is a sign that the cyst contents are dead; in this respect the *tetragena* cyst differs from the cyst of *E. coli*. Next, the periphery of the body becomes applied to the thin cyst membrane in the region where the wall is about to be dissolved (Fig. 4). Dissolution of the cyst-membrane takes place over a small area only. It is brought about, we consider, by the action of external influences; for example, the pancreatic juice, probably helped to some extent also by bacterial ferments present in the intestinal contents.

In the next stage (Fig. 5) the Entamoeba is beginning to emerge from the cyst. A small protuberance, often fingerlike, is thrust out through the aperture; it may appear clearer than the bulk of the protoplasm, and be mainly ectoplasmic in character. The whole of the protoplasm now gradually flows out of the cyst (Fig. 6); the entire process of emergence may take only about ten minutes, though it may take longer. In every instance which we have observed, the entire protoplasm comes out as a single Entamoeba; there is no indication whatever of division while still inside the cyst. We are strongly of the opinion that this is the normal method of excystation. It may be mentioned that tyrosin was always present to saturation in the liquor pancreaticus which we used, the white deposit which is usually seen at the bottom of a bottle of this preparation (after keeping for a while) consisting of pure tyrosin needles and sheaves which have crystallized out. We are informed by Dr. Maclean that the amount of this amino-acid normally present in the duodenum is not likely to represent a greater concentration than we have used in our tubes. From our observations we do not consider that a division into four amoebulae takes place, as a rule, within the cyst. After the Entamoeba has completely emerged, the cyst-membrane left behind appears like a very delicate, single-contoured, usually spherical ring (Fig. 7); only by careful focussing at a particular level can the break in the wall be made out, which represents the dissolved area (Fig. 8).

We have seen excystation of amoebae which had no chromidial block in the protoplasm (Fig. 6), as well as of others which possessed this structure (Fig. 8); so that one of these conditions cannot be said, apparently, to represent a riper phase, as regards readiness for excystation, than the other. For a little while the liberated Entamoeba remains more or less rounded and sluggish; but soon it begins to throw out the typical clear, blunt pseudopodia of the *histolytica* type, these often welling out laterally in the characteristic manner. We have not yet been able to carry out direct observations further at this period, and cannot say, therefore, whether the Entamoeba proceeds to divide into four, and, if so, how soon this occurs. Indeed, from our living observations, we could not tell whether the amoebae which excysted had always four nuclei, or whether individuals with only one or two nuclei also had been able to excyst.

When we have ascertained that excystation was proceeding actively in the tubes, we have always subcultured from them into the expression-liquid of blood-agar tubes, to which either two parts of citrated blood or equal parts of citrated blood and fresh serum were added. These new tubes were then placed in a vacuum and incubated at 37° C. At from eighteen to twenty hours after the excystation period we have seen both small and moderate-sized active *histolytica* forms in these cultures, a few of the larger individuals containing red blood cells. It is a moot point whether any multiplication had occurred, beyond probably division of some of the originally encysted forms into four; and even in regard to this we do not feel certain. In spite of all our efforts so far, the growth of bacteria has always been excessive and gave no promise of successful cultivation of the Entamoebae. On one or two occasions, twenty-four hours later—that is to say, from forty-two to forty-four hours after excystation—we have found one or two amoebae still alive but moribund.

To our surprise, the above method, which has proved so successful for the excystation of *E. histolytica*, has entirely failed to produce excystation of *E. coli*, and by none of the other methods tried have we been able to induce this form to excyst. We have evidence, however, that the small "*minuta*" form, which we have recently described,[1] excysts in the same manner as *histolytica* (*tetragena*), using the same method; and this bears out our view as to the close relationship of these two forms.

In the case of *Lamblia*, also, we have been unable to induce excystation. From the appearances of the cyst contents which we have observed, we are strongly of opinion that only a single individual will be liberated and not two separate individuals. In other words, we consider, as we have previously stated,[2] that conjugation and not division occurs within the *Lamblia* cyst. Wenyon[3] has expressed the view that these cysts are multiplicative, and represent, indeed, the endogenous form of multiplication shown by these parasites within the host. In our opinion this view is mistaken, and does not take into account the biological factors (environmental conditions) which influence both encystment and excystation (Cropper and Drew,[4] Woodcock and Lapage[5]). Not to enter into details here, we will only point out that when permanent (resistant) cysts, such as are passed out with the faeces, are formed in the bowel, conditions other than those prevailing in the region where encystment is occurring are necessary for excystation and the commencement of a fresh cycle of activity. Equally as is the case with the cysts of the parasitic Entamoebae, for instance, we have little doubt that these *Lamblia* cysts only undergo excystation on passing down the alimentary tract of a fresh host and coming into contact with its digestive juices. From our experience of multiplication in flagellates we should say the process takes place very rapidly; and it is quite likely that it occurs mainly amongst those parasites which are attached to the epithelium.

Had time permitted we had intended to use the above described method of inducing excystation of the cysts of *E. histolytica* to test the vitality, and consequently the infective power, of the cysts, when kept under different conditions for varying lengths of time; much more evidence is needed in regard to this point on account of its great hygienic and sanitary importance. The chief experiment which we have had opportunity to carry out, so far, in this direction has been to keep cysts in a very slowly running current of water for fifteen days and to ascertain that certainly some were still alive at the end of this period. By this time the débris was practically freed from faecal bacteria, apart from those normally occurring in the water, and free-living protozoa (ciliates and flagellates) were present. We should say, therefore, that water which has been contaminated with cyst-containing faeces may remain a source of infection for a considerable period.

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